

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
RAD: 12-144300- -7	FECHA: 2014-07-24 09:09:29
DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 12
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
CARLOS REINALDO OLARTE GARCIA

Asunto: Radicación: 12-144300- -7
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 8911
 Folios: 12

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/026016				
Fecha de Presentación Internacional	24/02/2011				

1. Documentos estudiados

Descripción:	Radicación 12-144300-00000	24/Agosto/2012
Reivindicaciones:	Radicación 12-144300-00000	24/Agosto/2012
Prioridades:	US 61/308,287	25/Febrero/2010
	US 61/406,541	25/Octubre/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 16 del capítulo reivindicatorio presentado mediante memorial N° Rad. 12-144300-00000-0000.

Título de la solicitud: “Cepa bacteriana aislada del género *Burkholderia* y metabolitos pesticidas del mismo”.

El problema planteado en esta solicitud consiste en la necesidad de encontrar alternativas de biocontrol específicamente de origen biológico a partir de cepas aisladas de *Burkholderia plantari*, *Burkholderia cepacea*, *Burkholderia gladioli*, *Burkholderia sp.* cuyos productos derivados del metabolismo presentan actividad pesticida.

Para resolver este problema técnico la solicitud en estudio proporciona especies de *Burkholderia sp.* sin patogenicidad conocida sobre vertebrados pero con actividad pesticida sobre insectos, hongos, malezas y nemátodos. La solicitud también proporciona los productos derivados del cultivo de la especie, específicamente compuestos con anillos de oxazol, tiazol e indol. La solicitud también proporciona los métodos para controlar la germinación y el crecimiento de dicotiledóneas, monocotiledóneas, malezas, hongos, insectos y nemátodos.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N1/20 // A01N43/14 // A01N63/02 // A01P7/00 // A01N43/74.

4. No es una invención (Art 15 D 486 literal (b))

El objeto de las reivindicaciones 1, 4, 5 y 6 no se considera una invención, de acuerdo con el Art citado, por cuanto corresponde a material biológico aislado de la naturaleza y al todo o parte de seres vivos tal y como se encuentra en la naturaleza, específicamente se define un microorganismo del género *Burkholderia* al que pertenecen otras cepas genéticamente cercanas como: *Burkholderia cepacia*, *Burkholderia plantari*, *Burkholderia gladioli*, *Burkholderia sp.*, *Burkholderia multivorans* y en general *Burkholderia sp.*, caracterizadas en términos de su ARN 16S y de los productos derivados de su metabolismo por referencia a técnicas de espectroscopía y cromatografía o por referencia general a su estructura. Se recuerda a la solicitante que no se consideran invenciones el todo o parte de seres vivos tal y como se encuentran en la naturaleza de acuerdo con el Art. 15 literal b) D 486.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (c))

El objeto de la reivindicación 16 no es patentable porque corresponde a la semilla sobre la cual se ha aplicado el producto de combinación agroquímica. Se recuerda a la solicitante que la Decisión 486 prohíbe el patentamiento de las plantas de acuerdo con el Art. 20 literal c).

6. Reivindicaciones relativas a un uso (Art 14 D 486)

El objeto de las reivindicaciones 14 y 15 no es patentable ya que aun cuando se encabezan en términos de método corresponden al uso y aplicación de los metabolitos o de composiciones que incluyen un sustrato biológico sobre una planta para evitar la infestación por plagas. Por otra parte, el uso de las reivindicaciones 17 y 18 no es una categoría patentable de acuerdo con el Art 14 D 486.

7. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no define de forma clara la invención porque:

- 1) Antepone al nombre científico de los microorganismos pertenecientes a la género *Burkholderia* la palabra “no”, lo cual no permite comprender el objeto y alcance de la invención, es decir, si corresponde simplemente a cepas aisladas de microorganismos genéticamente similares a los presentes en la naturaleza o a cepas genéticamente modificadas (recombinantes). El sustento de la materia reivindicada permite concluir que se trata de la cepa *Burkholderia sp* A396, sin embargo dicha cepa debe definirse en términos de sus características estructurales y de forma complementaria por su certificado de depósito de material biológico y no por referencia a diferencias frente a las especies de *Burkholderia cepacia*, *Burkholderia plantari*, *Burkholderia gladioli*, *Burkholderia sp*, *Burkholderia multivorans* y en general *Burkholderia sp*.
- 2) Por otra parte, la caracterización de la cepa en términos de las SEQ ID NO del rARN 16S según el literal a) de las reivindicaciones 1 y 2 tampoco puede considerarse elemento diferencial entre las cepas presentes en la naturaleza y la cepa *Burkholderia sp* A396, por cuanto en la descripción se indica que entre la cepa de interés y las presentes en la naturaleza existe un 99% de similitud y los conceptos básicos sobre Taxonomía indican que “*dos procariotas cuyo rARN 16S tengan un 97% o más de identidad entre las secuencias es muy probable que pertenezcan a la misma especie*”¹. Por lo tanto no se puede concluir con certeza que la materia reivindicada corresponda a un microorganismo diferente a los que se encuentran en la naturaleza.
- 3) En cuanto a la caracterización de la cepa en los términos de los literales b), c), d) y f) es decir por la capacidad pesticida y no patogénica frente a vertebrados de los metabolitos producidos por el microorganismo, caracterizados en términos de su espectro RMN y de la estructura funcional general, esto es porque i) son derivados de oxazolil-indol, oxazolil-bencilo, o por la presencia de grupos éster, amida, metileno o porciones de tetrahidropiranos, enlaces olefínicos, grupos metilo, hidroxilo y cadenas de al menos 25 carbonos, 8 oxígenos y un nitrógeno y ii) son ácidos grasos 16:0, ciclo 17:0, 16:0, 3-OH, 14:0, ciclo 19:0 ω8c, 18:0, es pertinente señalar que un microorganismo se define por la estructura de su genoma modificado y de forma complementaria por su certificado de depósito y no por los productos de su metabolismo porque además de no ser materia patentable en los términos del art. 15 lit. b) D 486 es decir porque forma parte del todo o parte de seres vivos como se encuentran en la naturaleza, tampoco se pueden identificar de forma inequívoca, dado que la información de dichos literales es de carácter funcional y omite definir la configuración estructural.

¹ Madigan, Michael T., Martinko John M. y Parker Jack. Biología de los Microorganismos. Octava Edición. Editorial Prentice Hall. España. 2000. Pág. 627.

- 4) Asimismo la información relativa a la susceptibilidad del microorganismo frente a antibióticos como: canamicina, cloramfenicol, ciprofloxacina, piperacilina, imipenem y combinaciones de sulfametoxazol y trimetoprim presente en el literal e) tampoco forma parte de los elementos característicos de la cepa debido a que corresponden a las ventajas y por ende, a los resultados que se pretenden alcanzar con la cepa mejorada.
- 5) No es claro porque la secuencia del ARNr de la cepa A396 se caracteriza por una identidad de al menos 99% frente a las secuencias ID 8, 11 y 12, pero dichas secuencias únicamente presentan una identidad de 91% (ID 8 y 11) y 82% (ID 8 y 12) al realizar el alineamiento respectivo. Se requiere a la Solicitante aclarar la metodología de alineamiento que emplea para establecer los porcentajes de identidad de la secuencia de ARNr 16S frente a las secuencias mencionadas.

Download Graphics Sort by: E value

Sequence ID: lc|44581 Length: 1152 Number of Matches: 2

Range 1: 292 to 841 Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
747 bits(404)	0.0	509/558(91%)	13/558(2%)	Plus/Plus
Query 286	GGGAGGCAGCAGTGGGGAAATTTTGGACAATGGGGGAAACCCCTGATCCAGCAATGCCGCGT	345		
Sbjct 292	GGGAGGCAGCTATGGGGAAATTTTGGACAATGTGGGAAACCCCTGATCCAACAATGCCGCGT	351		
Query 346	GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACATTTTGTCCGGAAGAAATCC-TTTGGGCT	404		
Sbjct 352	GTGTGAATAAGGCCTTCGGGTTGTAAAGCACATTTTATCCGGATAGA-TTCCTTTTGGGCT	410		
Query 405	AATACC-CCG-GGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGC	462		
Sbjct 411	AA-ACCTCCGTAGGGGATGACGGTACCGGAAGAATAACCACCGGTAACGTACGTGCCAGC	469		
Query 463	AGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGC	522		
Sbjct 470	AGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGC	529		
Query 523	AGGCGGTTTGTTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACGCAATTTGTGA	582		
Sbjct 530	AGGCGGTTTGTTAAGACAGATGTGAAATCCCCGGGCTTAACCTGGGAACGCAATTTGTGA	589		
Query 583	CTGGCAAGCTAGAGTATGGCAGAGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTA	642		
Sbjct 590	CTGGCAAGCTAGAGTATGGCAGAGGGGGTAGAATTCACGTGTAGCAGTGAAATGCGTA	649		
Query 643	GAGATGTGGAGGAATACCGATGG-CGAAGGCAGCCCCCTGGGCCAATACTGACGCTCATG	701		
Sbjct 650	GAGATGTGGAGGAATACCGATGGGCGAAG-CAGCT-CCTGGGGCAATACTGACGCTCATG	707		
Query 702	CACGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATG	761		
Sbjct 708	CACAAGATCGTGCGAAACAAACAGGATAA-AAACCCT-GTATTCCACGCCCAAACGATG	765		
Query 762	TCAACT-AGTTGTTGGGGATTTCATTCCTTAGTAACGTAGCTACGCGTGAAGTTGACCGC	820		

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Sequence ID: lcl|78117 Length: 1067 Number of Matches: 1

Range 1: 395 to 781 Graphics

Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
309 bits(167)	6e-88	328/402(82%)	26/402(6%)	Plus/Plus
Query 391	AAATCCTTTGGGCTAAT-ACCCCGGGGGGATGACGGTACCG-GAAGAATAAGCACCGGCT	448		
Sbjct 395	AAACCCCTTTGGCCTAATAACCCCGGGGGAAT-A-AGTACCGAAAAAATACTGGAT	452		
Query 449	AACT-ACGTGCCAGCAGCCGCGTAATACGTA--GGGTGCGAGCGTTAATCGGAA-TTAC	504		
Sbjct 453	AACTTCCGTGCCACAACCCGCGAAAAATCTAGGGGGGGGAGCTTAAATGGAAATTAC	512		
Query 505	TGGG-CGTAAAGCGTGCAGCAGCGGTTTGTAAAGACAGATGTGAAATCCCGGGCTTAAC	563		
Sbjct 513	GGGGCCGTAAAGCGTGCAGCAGCGGTTTGTAAACACAGATGTGAAATCCCGGGCTTAAC	572		
Query 564	CTGGGAAGTGCATTTGTGACTGGCAAGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACG	623		
Sbjct 573	CTGGGAAGTGCATTTGTGACTGGCAAGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACG	632		
Query 624	TGTAGCAGTGAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGG	683		
Sbjct 633	TGTAGCATTG-AATGCATAGAGATGAG-AGG-ATACCGATGGAGAAGGGCGCCCCCGGG	689		
Query 684	CCAATACTGACGCTCATGC-ACGAAAGC-GTGGGGAGCAAACAGGATTAGATACCCTG-G	740		
Sbjct 690	ACAATA-TGACGCCTATGCCAC-AAAGCTGTGGC-A-CAAT-AGG-TTAAATACC-TGTG	742		
Query 741	TAGTCCACGCCCTAAAC-GATGTCAACTAGTTGTTGGGGATT	781		
Sbjct 743	TTGTCCCCGCC-TAAACAGATTACA-CTTGTGTGT-GGGTATT	781		

La reivindicación 3 hace referencia a un medio de cultivo, se recuerda a la solicitante que la composición del medio de cultivo debe incluir la indicación de los nutrientes: oligoelementos, suplementos, entre otros y sus proporciones, condiciones de pH y la fase: sólida/líquida/semisólida que favorecen su crecimiento.

Las reivindicaciones 7 a 9 presentan inconsistencias en la medida que se encabezan en términos de Método pero se caracterizan por la estructura de los metabolitos producidos por el microorganismo de interés. Se recuerda a la solicitante que un método se caracteriza en términos de la secuencia lógica de etapas que permite sintetizar o aislar un compuesto activo, es decir, en términos de las reacciones o de las operaciones unitarias, sin embargo en el presente caso se definen compuestos que forman parte de las exclusiones a la patentabilidad dado que forman parte del todo o parte de seres vivos tal y como se encuentran en la naturaleza, de acuerdo con el art. 15 lit. b) D 486.

La reivindicación 10 se encabeza en términos de un método para aislar una cepa de *Burkholderia* caracterizado porque el microorganismo produce un metabolito y porque dicho producto de interés se aísla del sobrenadante. Al respecto es pertinente señalar que si bien corresponde a las etapas de un método, dicho método corresponde al aislamiento de un compuesto y no tiene como fin obtener y aislar una cepa específica del género *Burkholderia*.

Las reivindicaciones 11 a 13 se encabezan en términos de combinaciones. Sin embargo corresponden a composiciones que incluyen como ingrediente a) metabolitos aislados de la cepa *Burkholderia* o el cultivo puro, fracción celular o sobrenadante derivado de la cepa en combinación con b) un segundo agente agroquímico de tipo pesticida químico/biológico y c) un portador o diluyente. Por lo anterior se requiere que dichas reivindicaciones se encabecen en términos de composición y definan los ingredientes y proporciones por su estructura/nombre común/ de los compuestos combinados y por la presencia del cultivo puro

identificado en términos de su certificado de depósito y la SEQ ID NO que la diferencia de las cepas de *Burkholderia sp.* tal y como se encuentran en la naturaleza.

Descripción (Art 28 y 29 D 486)

Teniendo en cuenta que la solicitud reivindica material biológico aislado es necesario complementar la divulgación de la solicitud con el certificado de depósito de material biológico emitido por autoridad reconocida de conformidad con lo establecido en el Tratado de Budapest, específicamente para la cepa *Burkholderia sp* A396 a la que se hace referencia en el capítulo descriptivo y que fue depositada el 15 Septiembre 2009 en la Colección de Cultivos para Investigación Agrícola (NRRL) y cuyo número de acceso es NRRL B-50319.

8. Unidad de invención (Art 25 D 486)

A priori se encuentra que la solicitud no cumple con el requisito de unidad de invención previsto en el art. 25 D 486 por cuanto el único elemento técnico particular que comparten los grupos de invención corresponde a la presencia de la cepa aislada de *Burkholderia sp.*, la cual no se considera una invención en los términos del art. 15 lit. b) D 486.

En consecuencia, se encuentran por lo menos 3 grupos inventivos en las categorías de producto y proceso:

Grupo Inventivo 1: Medio de cultivo para el crecimiento de la cepa *Burkholderia sp.* A396, definido en la reivindicación 3.

Grupo Inventivo 2: Composiciones para el control biológico caracterizadas por la presencia de la cepa *Burkholderia sp.* A396 y un segundo ingrediente activo de tipo pesticida de origen químico o biológico y un portador/diluyente, definido en las reivindicaciones 11 a 13.

Grupo Inventivo 3: Métodos de aislamiento de un compuesto con actividad biológica/Método para aislar el material biológico del medio de cultivo, definido en las reivindicaciones 7 a 10.

El elemento común: cepa aislada de *Burkholderia sp.* se encuentra divulgado en el estado de la técnica de acuerdo con las enseñanzas del documento D1 (*Changes in agricultural management drive the diversity of Burkholderia species isolated from soil or PCAT medium* de Salles y colaboradores, 2005 y frente a Spilker T., et al.² que divulga una cepa de *Burkholderia multivorans* AU0127 caracterizada por un ARNr con un 99% de identidad frente a la cepa caracterizada por la SEQ ID No. 8). Por lo anterior, se sugiere a la solicitante presentar una o más de las divisionales propuestas, de acuerdo con los grupos de conceptos técnicos inventivos presentados sin ampliar el objeto inicial de la invención, limitar la solicitud a una sola invención eliminando de ella las otras invenciones o presentar las modificaciones que considere necesarias.

² PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. J Clin Microbiol. 2004 May;42(5):2074-9. <http://jcm.asm.org/content/42/5/2074.long>

9. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación internacional: febrero 25 de 2010, y se encontraron los siguientes documentos que anticipan el objeto de la solicitud.

Ítem	Documento	Título	Fecha de Publicación
D1	Salles y colaboradores.	“Changes in agricultural management drive the diversity of Burkholderia species isolated from soil o PCAT medium”	27/Julio/2005
D2	WO2007009224	“Natural herbicide from a <i>Burkholderia</i> cell-free culture fraction”	25/Enero/2007

El documento D1³ divulga un estudio sobre diversidad de poblaciones de *Burkholderia* en rizosfera y suelos, evaluando el impacto del régimen de manejo agrícola del suelo sobre la diversidad de las poblaciones (Pág. 662, Col. 1, Párr. 002). Las cepas fueron aisladas e identificadas empleando ARN 16S por medio de PCR-DGGE y BOX-PCR que permitió identificar 47 clusters diferentes (Pág. 66, Col. 1 y 2).

El documento D2⁴ divulga un herbicida que comprende una fracción libre de células del cultivo de especies de *Burkholderia* preferiblemente con un peso molecular inferior a 5000Da (Pág. 2, Párr. [008-010]). Asimismo, enseña un método para producir un compuesto herbicida que comprende obtener un cultivo libre de células de especies de *Burkholderia*, fraccionar dicho medio de cultivo condicionado y purificar un herbicida natural (Pág. 2, Párr. [015-016]). El documento enseña una composición que incluye la fracción o compuestos purificados, al menos un adyuvante (Rv. 10) y un herbicida adicional natural o sintético, donde dicho adyuvante es seleccionado de: surfactantes, viscosantes, colorantes, anti-espumantes, ente otros (Pág. 9, Párr. [036-038]).

10. Examen de Patentabilidad (Art 14 D486)
Novedad (Art 16 D 486)

Grupo Inventivo 1, Cultivo sustancialmente puro. (Reivindicación 3)

La reivindicación 3 consiste en “*Un cultivo sustancialmente puro, fracción celular o sobrenadante CARACTERIZADO porque se deriva de la cepa de conformidad con las reivindicaciones 1 – 2 o extracto de la misma*”

Características esenciales	D2
Cultivo sustancialmente puro, fracción celular o sobrenadante caracterizado porque	Una fracción libre de células del cultivo de especies de <i>Burkholderia</i> o un compuesto aislado del mismo caracterizado porque
se deriva de la cepa de <i>Burkholderia</i> sp de conformidad con las reivindicaciones 1 – 2 o extracto de la misma	se obtiene del cultivo de <i>Burkholderia</i> sp (Pág. 2, Párr. [008] a Pág. 3, Párr. [014])

3 <http://www.sciencedirect.com/science/article/pii/S0038071705002762>
 4 http://worldwide.espacenet.com/publicationDetails/claims?CC=WO&NR=2007009224A1&KC=A1&FT=D&ND=3&date=20070125&DB=EPODOC&locale=en_EP

Las características esenciales de la reivindicación 3 habían sido divulgadas previamente en el documento D2, el cual enseña una fracción libre de células del cultivo de especies de *Burkholderia* o un compuesto aislado del mismo que se obtiene del cultivo de *Burkholderia* sp (Pág. 2, Párr. [008] a Pág. 3, Párr. [014])

En consecuencia, la reivindicación 3 no es nueva o carece de novedad, según lo divulgado en el documento D2, toda vez que la cepa de *Burkholderia andropogonis* CW00B006C de D2 y la cepa de la reivindicación 1 definida por la SEQ ID No. 8, 11 y 12 presentan una identidad mayor al 95%, determinado por un alineamiento BLAST.

ref|NR_118985| (1435 letters)

RIDWDXUS663114 (Expires on 07-18 06:06 am)

Query IDgil645322176|ref|NR_118985.1|

DescriptionBurkholderia andropogonis strain ATCC 23061 16S ribosomal RNA gene, partial sequence

Molecule typenucleic acid

Query Length1435

Subject IDlcl|32451

DescriptionNone

Molecule typenucleic acid

Subject Length871

ProgramBLASTN 2.2.29+ [Citation](#)

Other reports: [Search Summary](#) [Taxonomy reports](#)

Sequence ID: lcl 32451 Length: 871 Number of Matches: 1					
Range 1: 1 to 846 Graphics Next Match Previous Match					
Score	Expect	Identities	Gaps	Strand	
1336 bits(723)	0.0	806/848(95%)	4/848(0%)	Plus/Plus	
Query 25	TGCAAGTCGAACGCTAACAC-GGGAGCTTGCTCC-GCTGACGAGTGGCGAACGGGTGAGTA	82			
Sbjct 1	TGC-AGTCGAACGCGACGCGGTGCTTGCACTGGTGGCGAGTGGCGAACGGGTGAGTA	59			
Query 83	ATACATCGGAACGTGTCTGTAGTGGGGGATAGCCCGCGAAGCINNATTAAATACCGCA	142			
Sbjct 60	ATACATCGGAACATGTCTGTAGTGGGGGATAGCCCGCGAAGCOCGATTAAATACCGCA	119			
Query 143	TACGATCTAAGGATGAAAGCGGGGGATCTTCGGACTCGCGCTATAGGGCGGGCGATGG	202			
Sbjct 120	TACGATCTACGGATGAAAGCGGGGGATCTTCGGACTCGCGCTATAGGGTTGGCGGATGG	179			
Query 203	CAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAGCTGGTCTGAG	262			
Sbjct 180	CTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCTGGTCTGAG	239			
Query 263	AGGACGACCCACACACTGGGACTGAGACACGGCCACAGCTCTACGGGAGGCAGCAGTG	322			
Sbjct 240	AGGACGATCAGCCACACTGGGACTGAGACACGGCCACAGCTCTACGGGAGGCAGCAGTG	299			
Query 323	GGGAATTTTGGACAATGGGCGCAAGCCTTGATCCAGCAATGCCGCGTGTGTGAAGAGGCC	382			
Sbjct 300	GGGAATTTTGGACAATGGGCGAACCCTTGATCCAGCAATGCCGCGTGTGTGAAGAGGCC	359			
Query 383	TTGGGTTGTAAAGCACTTTTGTTCGGGAAGAAAGGCGAGGGCTAATATCCTTTGCTGA	442			
Sbjct 360	TTGGGTTGTAAAGCACTTTTGTTCGGGAAGAAATCCTTTTGGGCTAATACCCCGGGGGA	419			
Query 443	TGACGGTACCGGAAGATAAGCACCGGCTAATACGTGCCAGCAGCCCGGTAAATACGTA	502			
Sbjct 420	TGACGGTACCGGAAGATAAGCACCGGCTAATACGTGCCAGCAGCCCGGTAAATACGTA	479			
Query 503	GGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTAAAGAC	562			
Sbjct 480	GGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTGTAAAGAC	539			
Query 563	CGATGTGAAATCCCGGGCTTAACCTGGGAACTGCAATTTGTGACTGGCAGGCTAGAGTGT	622			
Sbjct 540	AGATGTGAAATCCCGGGCTTAACCTGGGAACTGCAATTTGTGACTGGCAAGCTAGAGTAT	599			
Query 623	GGCAGAGGGGGTAGAATTCGCGGTAGCAGTGAATGCGTAGATATGTGGAGGAATAC	682			
Sbjct 600	GGCAGAGGGGGTAGAATTCACGTGTAGCAGTGAATGCGTAGAGATGTGGAGGAATAC	659			
Query 683	CGATGGCGAAGGCGAGCCCTGGGCGCAATCTGACGCTCATGCAAGAAAGCGTGGGGAGC	742			
Sbjct 660	CGATGGCGAAGGCGAGCCCTGGGCGCAATCTGACGCTCATGCAAGAAAGCGTGGGGAGC	719			
Query 743	AAACAGGATTAGATACCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTGGGGA	802			
Sbjct 720	AAACAGGATTAGATACCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTGGGGA	779			
Query 803	TTCAITTCCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCTGGGGAGTACGGTCCGA	862			
Sbjct 780	TTCAITTCCTTAGTAACGTAGCT-AACGCGTGAAGTTGACCGCTGGGGAGTACGGTCCGA	838			
Query 863	AGATTAAA 870				
Sbjct 839	AGATTAAA 846				

Grupo Inventivo 3, Métodos de aislamiento de un compuesto con actividad biológica/Método para aislar el material biológico del medio de cultivo (Reivindicación 7)

La reivindicación 7 consiste en: “un método para producir un compuesto caracterizado porque se selecciona del grupo que consiste de: (A)...en el cual el método comprende cultivar la cepa de conformidad con las reivindicaciones 1-2 y producir el compuesto.

Características esenciales	D2
un método para producir un compuesto caracterizado porque se selecciona del grupo que consiste de: (A)...en el cual el método comprende:	Método para producir un compuesto herbicida que comprende:
cultivar la cepa de conformidad con las reivindicaciones 1-2 y producir el compuesto.	obtener un cultivo libre de células de especies de <i>Burkholderia</i> , fraccionar dicho medio de cultivo condicionado y purificar un herbicida natural (Pág. 2, Párr. [015-016]).

Las características esenciales de la reivindicación 7 habían sido divulgadas previamente en el documento D2, el cual enseña un método para producir un compuesto herbicida que comprende obtener un cultivo libre de células de especies de *Burkholderia*, fraccionar dicho medio de cultivo condicionado y purificar un herbicida natural (Pág. 2, Párr. [015-016]).

En consecuencia, la reivindicación 7 no es nueva o carece de novedad, según lo divulgado en el documento D2, toda vez que la cepa de *Burkholderia andropogonis* CW00B006C de D2 y la cepa de la reivindicación 1 definida por la SEQ ID No. 8, 11 y 12 presentan una identidad mayor al 95%, determinado por un alineamiento BLAST.

Grupo Inventivo 2: Composiciones para el control biológico caracterizadas por la presencia de la cepa *Burkholderia sp.* A396 y un segundo ingrediente activo de tipo pesticida de origen químico o biológico y un portador/diluyente, definido en las reivindicaciones 11 a 13.

La reivindicación 11 consiste en: “una combinación caracterizada porque comprende (a) una primer sustancia seleccionada del grupo que consiste de (i) un cultivo puro, fracción celular o sobrenadante de la cepa de las reivindicaciones 1-2 o extracto de la misma, (ii) el compuesto de las reivindicaciones 4 a 6; (iii) un compuesto producido por la cepa de las reivindicaciones 1-2 y (b) un compuesto aislado; (b) opcionalmente una segunda sustancia, seleccionado de un pesticida químico o biológico y (c) opcionalmente al menos un portador, diluyente, tensioactivo, adyuvante.

Características esenciales	D2
Combinación caracterizada porque comprende:	Composición que contiene (Rv. 10):
(a) una primer sustancia seleccionada del grupo que consiste de (i) un cultivo puro, fracción celular o sobrenadante de la cepa de las reivindicaciones 1-2 o extracto de la misma, (ii) el compuesto de las reivindicaciones 4 a 6; (iii) un compuesto producido por la cepa de las	(a) una fracción libre de células del cultivo de especies de <i>Burkholderia</i> o compuestos purificados de las mismas,

Características esenciales	D2
reivindicaciones 1-2	
(b) opcionalmente una segunda sustancia, seleccionado de un pesticida químico o biológico	(b) un herbicida adicional natural o sintético,
(c) opcionalmente al menos un portador, diluyente, tensioactivo, adyuvante.	(c) un adyuvante seleccionado de: surfactantes, viscosantes, colorantes, anti-espumantes, ente otros (Pág. 9, Párr. [036-038]).

Las características esenciales de la reivindicación 11 habían sido divulgadas previamente en el documento D2, el cual enseña una composición que incluye una fracción libre de células del cultivo de especies de *Burkholderia* o compuestos purificados de las mismas, al menos un adyuvante (Rv. 10) y un herbicida adicional natural o sintético, donde dicho adyuvante es seleccionado de: surfactantes, viscosantes, colorantes, anti-espumantes, ente otros (Pág. 9, Párr. [036-038]).

En consecuencia, la reivindicación 11 no es nueva o carece de novedad, según lo divulgado en el documento D2, toda vez que la cepa de *Burkholderia andropogonis* CW00B006C de D2 y la cepa de la reivindicación 1 definida por la SEQ ID No. 8, 11 y 12 presentan una identidad mayor al 95%, determinado por un alineamiento BLAST.

De la misma forma, las reivindicaciones dependientes 12 y 13 no aportan características adicionales que hagan novedosa dicha composición de la reivindicación 11.

11. Conclusión

Los requisitos de patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	WO2007009224	3, 7 y 11 a 13	Novedad

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JESUS FERNEL GARCÍA GARCÍA
Director de Nuevas Creaciones (E)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-07-29

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**PCR-Based Assay for Differentiation of
Pseudomonas aeruginosa from Other
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PCR-Based Assay for Differentiation of *Pseudomonas aeruginosa* from Other *Pseudomonas* Species Recovered from Cystic Fibrosis Patients

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Pseudomonas aeruginosa is the major opportunistic bacterial pathogen in persons with cystic fibrosis (CF); pulmonary infection occurs in approximately 80% of adult CF patients. Much of CF patient management depends on accurate identification of *P. aeruginosa* from sputum culture. However, identification of this species may be problematic due to the marked phenotypic variability demonstrated by CF sputum isolates and the presence of other closely related species. To facilitate species identification, we used 16S ribosomal DNA (rDNA) sequence data to design PCR assays intended to provide genus- or species-level identification. Both assays yielded DNA fragments of the predicted size. We tested 42 culture collection strains (including 14 *P. aeruginosa* strains and 28 strains representing 16 other closely related *Pseudomonas* species) and 43 strains that had been previously identified as belonging to 28 nonpseudomonal species also recovered from CF patient sputum. Based on these 85 strains, the specificity and sensitivity of both assays were 100%. To further assess the utility of the PCR assays, we tested 66 recent CF sputum isolates. The results indicated that preliminary phenotypic testing had misidentified several isolates. The 16S rDNA sequence was determined for 38 isolates, and in all cases it confirmed the results of the PCR assays. Thus, we have designed two PCR assays: one is specific for the genus *Pseudomonas*, while the other is specific for *P. aeruginosa*. Both assays show 100% sensitivity and specificity.

Pseudomonas aeruginosa is the most common bacterial pathogen causing respiratory tract infection in persons with cystic fibrosis (CF). Infection occurs throughout childhood, ultimately affecting some 80% of adult CF patients, and is associated with increased rates of morbidity and mortality (9, 13). *P. aeruginosa* is also a well-known opportunistic and nosocomial pathogen, the identification of which typically presents little challenge. Accurate identification of CF isolates, however, can be difficult. CF-derived isolates often demonstrate phenotypic diversity due to loss of pigment production, exopolysaccharide production (mucoidy), and synthesis of rough lipopolysaccharide (23). Commercial test systems and other phenotype-based identification methods may therefore misidentify *P. aeruginosa* (15, 18, 28, 29, 31). Identification is often further hampered by the presence of other closely related nonfermenting gram-negative bacilli, including other *Pseudomonas* species (2, 6). The potential for misidentification of this species from sputum culture presents an obstacle to CF patient management, particularly with respect to antimicrobial therapy, patient prognosis, and infection control.

Genotype-based identification methods circumvent the problem of variable phenotype to provide more accurate species identification. However, the taxonomic complexity, uncertain phylogeny, and paucity of genomic sequence data of the dozens of species within the broad genus *Pseudomonas* present an obstacle to genotypic identification assays. We took advantage of a recent comprehensive reassessment of the phylogenetic affiliation of the pseudomonads based on 16S ribosomal

DNA (rDNA) sequence data (1) to identify genus- and species-specific 16S rDNA signature sequences. Based on these sequences we designed simple, rapid, and accurate PCR assays that allow the differentiation of *P. aeruginosa* from other *Pseudomonas* species that may also be recovered from CF sputum cultures.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Forty-two *Pseudomonas* strains were obtained from the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium) or the American Type Culture Collection (Manassas, Va.). This included 14 *P. aeruginosa* strains and at least one strain each of 16 other *Pseudomonas* species (Table 1; Fig. 1). Another 43 strains had been identified in previous studies as belonging to 28 nonpseudomonal species (4–8, 21, 22, 24, 33). This group included 15 *Burkholderia cepacia* complex, 5 *Pandoraea* spp., 5 *Ralstonia* spp., 5 *Achromobacter xylosoxidans*, 4 *Stenotrophomonas maltophilia*, 2 *Acinetobacter* spp., and 2 *Serratia marcescens* strains and 1 strain each of *Herbaspirillum frisingense*, *Klebsiella pneumoniae*, *Morganella morganii*, *Moraxella osloensis*, and *Escherichia coli*. An additional 66 isolates (recovered from 66 CF patients) were selected from isolates referred to the *Burkholderia cepacia* Research Laboratory and Repository (BcRLR, University of Michigan) for analysis. This group consisted of isolates that had been referred to the BcRLR identified as either *P. aeruginosa* ($n = 14$ isolates) or another *Pseudomonas* species ($n = 20$). It also included isolates that had been unidentified ($n = 12$) or identified by the referring laboratory as a nonpseudomonal species ($n = 20$) but which were identified by the BcRLR as a *Pseudomonas* species by using the RapID NF Plus system (Remel, Lenexa, Kans.). All bacteria were stored at -80°C . Bacteria from frozen stocks were grown aerobically at 34°C for as long as 48 h on Mueller-Hinton medium supplemented with 1.6% (wt/vol) agarose.

DNA preparation. DNA was prepared from bacteria as described previously (22). In brief, a single CFU was suspended in 20 μl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 N NaOH. After heating for 15 min at 95°C , 180 μl of high-performance liquid chromatography-grade H_2O (Fisher) was added, and the lysis suspension was stored at -20°C .

Primer design. Relevant 16S rDNA sequences available in the GenBank database were aligned by using the MegAlign software package (DNASTAR Inc., Madison, Wis.). These included 136 sequences from 42 validly described

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TABLE 1. Culture collection *Pseudomonas* strains used in this study

Species	Strain ^a
<i>P. aeruginosa</i>	ATCC 10145, ATCC 14207, ATCC 19154, ATCC 19429, ATCC 23993, ATCC 25006, ATCC 25010, ATCC 25619, ATCC 27315, ATCC 27316, ATCC 35032, ATCC 35422, ATCC 35554, ATCC 9721
<i>P. agarici</i>	LMG 2112T
<i>P. alcaligenes</i>	LMG 1224T, LMG 6353
<i>P. chlororaphis</i>	LMG 5004T
<i>P. fluorescens</i>	LMG 18387T
<i>P. fluorescens</i>	LMG 14564, LMG 1794T, LMG 5940
<i>P. fulva</i>	LMG 11722T
<i>P. fuscovaginae</i>	LMG 2158T
<i>P. mendocina</i>	LMG 1223T, LMG 5941, LMG 6396
<i>P. oleovorans</i>	LMG 2229T
<i>P. pertucinogena</i>	LMG 1874T
<i>P. pseudoalcaligenes</i>	LMG 1225, LMG 2854, LMG 5516
<i>P. putida</i>	LMG 2257T, LMG 2259
<i>P. resinovorans</i>	LMG 2274T
<i>P. stutzeri</i>	ATCC 17588, LMG 11199T, LMG 1228, LMG 2232
<i>P. syringae</i>	LMG 1247T, LMG 12648
<i>P. tolaasii</i>	LMG 2342

^a ATCC, American Type Culture Collection, Manassas, Va.; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

Pseudomonas species (1), as well as several other phylogenetically related γ -*Proteobacteria* and CF-relevant species. Based on this alignment, putative genus- and species-specific primers were designed.

PCR. Amplification of targeted DNA was carried out in 25- μ l reaction volumes, each containing 2 mM MgCl₂, 50 mM Trizma (pH 8.3; Sigma, St. Louis, Mo.), 250 μ M (each) deoxynucleoside triphosphates (Promega, Madison, Wis.), 0.4 μ M (each) primer, 1 U of *Taq* polymerase (Invitrogen, Carlsbad, Calif.), and 2 μ l of whole-cell bacterial lysate, and adjusted to 25 μ l by the addition of high-performance liquid chromatography-grade H₂O. Amplification was carried out in a RapidCycler (Idaho Technology Inc., Salt Lake City, Utah) thermocontroller. After an initial denaturization for 2 min at 95°C, 25 cycles were completed, each consisting of 20 s at 94°C, 20 s at the appropriate annealing tem-

perature (Table 2), and 40 s at 72°C. A final extension of 1 min at 72°C was applied. With this program, the total time for amplification of target DNA was approximately 45 min.

Amplification and sequence determination of 16S rDNA. In order to confirm PCR-based identification results, we performed comparative 16S rDNA sequence analysis. Nearly complete 16S rRNA genes (corresponding to positions 9 to 1500 in the *E. coli* numbering system) were amplified by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) with conserved primers UFPL and URPL as previously described (22). Amplified DNA was purified with the QIAquick PCR purification kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's instructions. DNA sequencing was carried out with an Applied Biosystems ABI model 3700 sequencer and the protocols of the manufacturer (PE

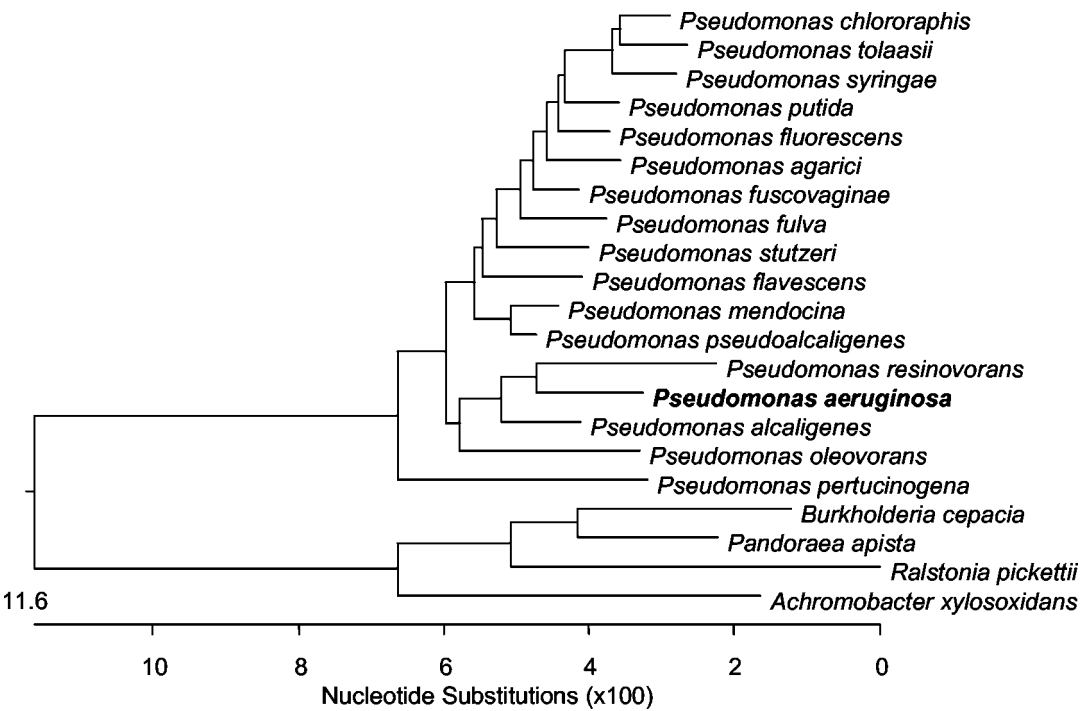


FIG. 1. Phylogenetic tree generated by using ClustalV-based alignment for 16S rDNA sequences of select *Pseudomonas* and other CF-related species. *P. resinovorans*, *P. aeruginosa*, *P. alcaligenes*, and *P. oleovorans* are species within the *P. aeruginosa* group (1).

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TABLE 2. 16S rDNA-based primer sets

Primer	Sequence (5'-3')	Target	Annealing temp (°C)	Location ^a	Product size (bp)
PA-GS-F	GACGGGTGAGTAATGCCTA	<i>Pseudomonas</i> species	54	95–113	618
PA-GS-R	CACTGGTGTTCCTTCCTATA			693–712	
PA-SS-F	GGGGGATCTTCGGACCTCA	<i>P. aeruginosa</i>	58	189–206	956
PA-SS-R	TCCTTAGAGTGCCACCCG			1124–1144	

^a Position and size relative to 16S rDNA sequence of *P. aeruginosa* AT2 (AB091760).

Applied Biosystems, Foster City, Calif.) by using the BigDye Terminator cycle sequencing ready reaction kit. Resultant sequences were visualized as chromatograms and manually edited using Chromas version 2.22 (Technelysium Pty. Ltd., Helensvale, Australia). Edited sequences were assembled using EditSeq (DNASTAR Inc.) and identified by using BLASTN and comparison to sequences currently available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST). Isolates were considered *P. aeruginosa* if their 16S rDNA sequences showed the highest degree of similarity to 16S rDNA sequences of *P. aeruginosa* culture collection reference strains.

Nucleotide sequence accession number. The GenBank accession numbers for the 16S rDNA sequences generated in this study are AY486350 through AY486387.

RESULTS

Primer design. Based on alignment of 16S rDNA sequences available in GenBank, two primer pairs were designed. Primer pair PA-GS-F and PA-GS-R was intended to amplify all *Pseudomonas* species, while the pair PA-SS-F and PA-SS-R was designed to amplify only *P. aeruginosa* (Table 2). The latter primers targeted species-specific signature sequences in 16S rDNA variable regions 2 and 8 (V2 and V8), respectively.

Sensitivity and specificity of PCR assays. PCR assays employing each primer pair produced DNA products of the predicted sizes (Fig. 2). The sensitivity and specificity of the putative genus-specific and species-specific PCR assays were determined by testing the 42 culture collection *Pseudomonas* strains (Table 1; Fig. 1) and the 43 previously identified non-*Pseudomonas* strains. The results are summarized in Table 3; the sensitivity and specificity of each PCR assay were 100%.

Further assessment of PCR assays. As another test of the utility of the two new PCR assays, we investigated 66 sputum culture isolates (recovered from 66 CF patients) that had been

referred to the BcRLR for analysis. The results are summarized in Table 4. Fourteen isolates in this group were identified by the referring laboratories as *P. aeruginosa*. Thirteen of these were also identified as *P. aeruginosa* by PCR assay; 16S rDNA sequence analysis was performed on six of these, which confirmed all as *P. aeruginosa*. The single isolate that was not a *Pseudomonas* species by PCR assay was identified as a *Burkholderia* species by 16S rDNA sequence analysis.

Twenty isolates had been identified by the referring laboratory as either “*Pseudomonas* sp.” ($n = 10$), *P. alcaligenes* ($n = 1$), *P. mendocina* ($n = 2$), *P. oleovorans* ($n = 1$), *P. fluorescens* ($n = 3$), *P. putida* ($n = 2$), or *P. stutzeri* ($n = 1$). Seventeen of these were identified as *P. aeruginosa* by PCR assay; 16S rDNA sequence analysis of six of these confirmed that they were *P. aeruginosa*. One isolate was identified as a non-aeruginosa pseudomonal species by PCR and confirmed as *P. synxantha* by 16S rDNA analysis. The remaining two isolates were not *Pseudomonas* sp. by PCR and were identified as *Burkholderia* sp. and *S. maltophilia* by 16S rDNA sequence analysis.

Twenty isolates were identified by referring laboratories as species other than *Pseudomonas* sp. Twelve of these were identified as *P. aeruginosa* by PCR; six were tested by 16S rDNA sequence analysis and confirmed as *P. aeruginosa*. Five of the 20 isolates were identified by both PCR assay and 16S rDNA analysis as non-aeruginosa pseudomonal species. Three isolates were not *Pseudomonas* sp. by PCR and were identified as *Herbaspirillum* sp. by 16S rDNA analysis. The remaining 12 isolates were unidentified by the referring laboratories. Eight of these were identified as *P. aeruginosa* by PCR assay; four were examined by 16S rDNA and confirmed as *P. aeruginosa*.

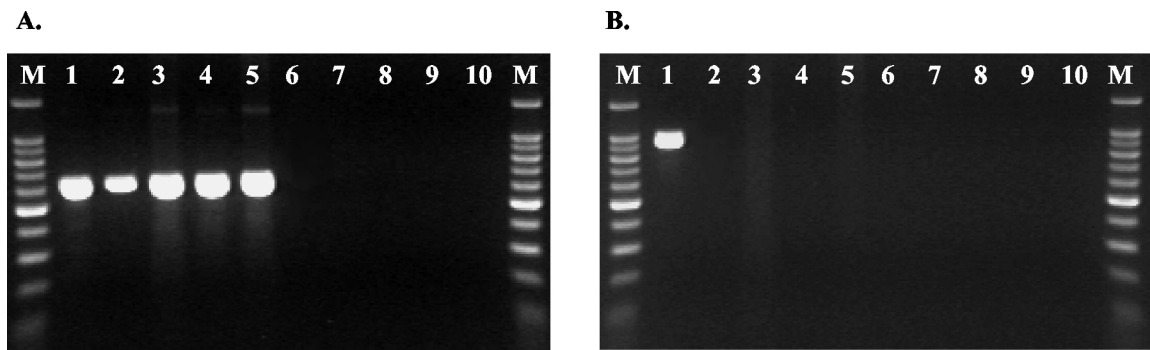


FIG. 2. PCR analysis of *P. aeruginosa*, *Pseudomonas* species, and CF-relevant bacteria. (A) PCR using *Pseudomonas* genus-specific primers PA-GS-F and PA-GS-R. (B) PCR using *P. aeruginosa*-specific primers PA-SS-F and PA-SS-R. Lanes M, reference markers; lane 1, *P. aeruginosa*; lanes 2 to 5, *P. pertucinogena*, *P. stutzeri*, *P. putida*, and *P. syringae*, respectively; lanes 6 to 9, CF-relevant genera (*Burkholderia*, *Pandoraea*, *Ralstonia*, and *Achromobacter*, respectively); lane 10, negative control (water).

TABLE 3. Sensitivity and specificity of PCR assays

PCR using primer pair	Target	No. of PCR positive/no. tested			Sensitivity (%)	Specificity (%)
		<i>Pseudomonas</i> sp. ^a	<i>P. aeruginosa</i> ^b	Other ^c		
PA GS-F and PA GS-R	<i>Pseudomonas</i> species	28/28	14/14	0/43	100	100
PA SS-F and PA SS-R	<i>P. aeruginosa</i>	0/28	14/14	0/43	100	100

^a Culture collection *Pseudomonas* species other than *P. aeruginosa* (detailed in Table 1).

^b Culture collection *P. aeruginosa* only (detailed in Table 1).

^c Previously identified non-*Pseudomonas* species recovered from CF sputum (includes 15 *B. cepacia* complex, 5 *Pandora* sp., 5 *Ralstonia* sp., 5 *A. xylosoxidans*, 4 *S. maltophilia*, 2 *Acinetobacter* sp., and 2 *S. marcescens* strains and 1 strain each of *H. frisingense*, *K. pneumoniae*, *M. morgani*, *M. osloensis*, and *E. coli*).

PCR assays identified two isolates as non-aeruginosa pseudomonal species and two as not *Pseudomonas* species; 16S rDNA sequence analysis of these four isolates confirmed the PCR results.

In summary, the novel species- and genus-specific PCR assays indicated that several of the 66 clinical isolates had been misidentified by the referring laboratories. Thirty-eight (58%) of these were further examined by 16S rDNA sequence analysis, and in each case the results were consistent with the results of the PCR assays. Thus, when assessed against 16S rDNA sequence analysis of this set of isolates, the sensitivity and specificity of both PCR assays were again 100%.

DISCUSSION

Persons with CF are susceptible to chronic respiratory tract infection by a number of opportunistic bacterial pathogens, including *P. aeruginosa*, *S. maltophilia*, *A. xylosoxidans*, and several *Ralstonia*, *Pandora*, and *B. cepacia* complex species (2, 20). Recent work has shown that CF patients also can become infected with other unusual or novel taxa, such as *Acinetobacter* sp., *Bordetella* sp., *Moraxella* sp., *Comomonas* sp., *Chryseobacterium* sp., *Rhizobium* sp., *Herbaspirillum* sp., and *Inquilinus limosus* (6). Accurate identification of these phenotypically similar species from respiratory samples is critical to patient management. This is particularly true with respect to *P. aeruginosa*. Sustained infection by this species is typically regarded as a poor prognostic indicator in young CF patients (13,

19). In addition, recent evidence of interpatient spread of *P. aeruginosa* among CF patients (14) has prompted increasingly stringent infection control measures in CF care centers; the effectiveness of such measures relies in the first instance on accurate species identification. The differentiation of *P. aeruginosa* from other *Pseudomonas* species is also fundamental to efforts to better understand the natural history of *P. aeruginosa* infection in CF and to develop and assess new therapeutic strategies (e.g., aggressive antimicrobial therapy aimed at eradication of initial pulmonary infection) (19). In fact, entry into clinical trials evaluating novel therapies relies on the accurate and rapid identification of *P. aeruginosa* and other infecting species.

The CF lung, however, constitutes a microenvironment that promotes phenotypic alteration of chronically infecting bacteria (26). Consequently, the atypical phenotypes often exhibited by *Pseudomonas* CF isolates present a challenge to commercial test systems commonly used in clinical microbiology laboratories (15, 18, 28, 29, 31). Genotypic identification methods would be expected to circumvent this problem, and molecular assays based on specific genes have been described. These include PCR assays targeting the *P. aeruginosa* *oprL* (11), *algD* (10, 25), and exotoxin A genes (17, 32). The performance of these assays in differentiating *P. aeruginosa* from other closely related *Pseudomonas* species has not been examined, however, and the paucity of sequence data for these loci from non-*P. aeruginosa* strains limits predictions based on in silico analyses. More recently, Clarke and colleagues (3) described a PCR assay that amplifies a fragment of the *groE* heat shock protein gene from several *Pseudomonas* species. Restriction fragment length polymorphism analysis of the amplified DNA was reported to differentiate *P. aeruginosa* from *P. stutzeri*, *P. fluorescens*, and *P. putida*. Qin and colleagues (28) used real-time PCR amplification of multiple targets, including exotoxin A, *algD*, *oprL*, and *gyrB*, together with biochemical tests and 16S rDNA sequencing to identify phenotypically atypical *P. aeruginosa* isolates recovered from CF specimens.

16S rDNA sequence has long been used as a taxonomic "gold standard" in determining the phylogenies of bacterial species (35). Selective amplification of *Pseudomonas* 16S rDNA by PCR followed by restriction fragment length polymorphism analysis or denaturing gradient gel electrophoresis has been used to detect and differentiate *Pseudomonas* species from clinical and environmental samples (12, 27, 30, 34). Karpatis and Jonasson (16) used a conserved 16S rDNA primer with a *Pseudomonas* genus-specific primer in a PCR assay to detect *Pseudomonas* DNA in CF sputum; this assay was not

TABLE 4. PCR analysis of CF sputum isolates referred to BcRLR

Referring lab identification ^a	PCR analysis ^b		
	<i>P. aeruginosa</i>	<i>Pseudomonas</i> sp. (non-aeruginosa)	Not <i>Pseudomonas</i> sp.
<i>P. aeruginosa</i>	13 (6 ^c)		1 (1 ^d)
<i>Pseudomonas</i> sp. (non-aeruginosa)	17 (6 ^c)	1 (1 ^e)	2 (2 ^f)
Not <i>Pseudomonas</i> sp.	12 (6 ^c)	5 (5 ^g)	3 (3 ^h)
No identification	8 (4 ^c)	2 (2 ⁱ)	2 (2 ^j)

^a Identification provided by referring laboratory.

^b Results obtained with PCR assays using species-specific primers PA SS-F and PA SS-R and genus-specific primers PA GS-F and PA GS-R. Numbers in parentheses indicate number of isolates for which 16S rDNA sequence analysis was performed. Superscript, italic letters c through j indicate species tentatively identified by 16S rDNA sequence analysis as follows: c, *P. aeruginosa*; d, *Burkholderia* sp.; e, *P. synxantha*; f, *Burkholderia* sp. and *S. maltophilia*; g, *P. fluorescens*, *P. stutzeri*, *P. lundensis/fragi*, and two *Pseudomonas* sp.; h, *H. seropedicae* and two *Herbaspirillum* sp.; i, *P. lundensis/fragi* and *P. pseudoalcaligenes*; j, *Staphylococcus* sp. and *Acinetobacter* sp.

designed to differentiate *P. aeruginosa* from other *Pseudomonas* species.

In this study, we took advantage of a recent reassessment of the phylogenetic affiliation of the pseudomonads (1) to reexamine the rapidly expanding 16S rDNA sequence data available in public databases. Based on an alignment of 136 16S rDNA sequences from 42 validly described *Pseudomonas* species as well as several other γ -Proteobacteria, we identified *Pseudomonas* genus-specific and *P. aeruginosa*-specific signature sequences. PCR assays using primers targeting these sequences were designed and tested against a panel of 85 previously identified strains. We included in this test panel 43 strains representing 28 nonpseudomonal species that are also found in CF sputum, as well as 42 culture collection *Pseudomonas* strains. In the latter group we were careful to include strains from *Pseudomonas* species phylogenetically most closely related to *P. aeruginosa*. This group included *P. resinovorans*, *P. alcaligenes*, *P. oleovorans*, *P. pseudoalcaligenes*, *P. mendocina*, and *P. fluorescens*, all of which cluster within the *P. aeruginosa* group described by Anzai and colleagues (1). We also tested our PCR assays against *P. putida*, *P. fluorescens*, *P. stutzeri*, and *P. syringae*, common environmental pseudomonads that may also be recovered occasionally from CF sputum. Both the genus-specific and *P. aeruginosa*-specific assays demonstrated 100% sensitivity and specificity.

Our examination of recent CF sputum isolates further showed the utility of the new PCR assays. Sixty-six isolates, recovered from 66 patients and referred from several clinical microbiology laboratories, were examined. All had been assessed phenotypically with a commercial test system by the referring laboratory. Among these, 34 had been identified by the referring laboratory as either *P. aeruginosa*, another *Pseudomonas* species, or as *Pseudomonas* (i.e., not identified to the species level). The remaining 32 had been referred either unidentified or identified as a nonpseudomonal species but were preliminarily identified by us as *Pseudomonas* using our routine phenotypic test kit (RapID NF Plus system). Analysis of these 66 isolates with the two novel PCR assays indicated that phenotypic testing had misidentified several isolates. We performed 16S rDNA sequence analysis on more than half of these isolates, and in every case the sequence data confirmed the PCR results.

Although our PCR and DNA sequence analyses revealed isolates that had been misidentified by phenotypic testing, we must be clear in pointing out that our study was not designed to ascertain the frequency of misidentification of CF sputum isolates nor to compare the relative accuracy of different phenotypic identification systems. Isolates referred to us for testing most likely represent a biased set of atypical and difficult-to-identify isolates, and so extrapolation of misidentification rates from this study is not appropriate. Nevertheless, such isolates were well suited to provide a rigorous test of our new PCR assays and represented strains for which molecular analysis would be expected to be most useful. Our study also reiterates that various non-aeruginosa pseudomonal species can occasionally be recovered from CF sputum culture. Among isolates testing positive with the genus-level PCR, but negative with the *P. aeruginosa*-specific PCR, we identified *P. fluorescens*, *P. lundensis*/fragi *P. pseudoalcaligenes*, *P. stutzeri*, and *P. synxantha*, based on 16S rDNA sequence analysis.

In summary, we have designed 16S rDNA-based PCR assays that provide rapid, simple, and reliable identification of *P. aeruginosa* and its differentiation from other phylogenetically closely related *Pseudomonas* species. Both assays have 100% sensitivity and specificity for their intended targets. We have also demonstrated the utility of these PCR assays in accurately identifying *P. aeruginosa* among isolates not correctly identified by phenotypic analyses. These assays should serve as a useful adjunct in the evaluation of gram-negative nonfermenting bacteria recovered from CF sputum culture.

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Changes in agricultural management drive the diversity of *Burkholderia* species isolated from soil on PCAT medium

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Abstract

In order to assess the diversity of culturable *Burkholderia* populations in rhizosphere and bulk soil and to evaluate how different agricultural management regimes and land use history affect this diversity, four treatments were evaluated: permanent grassland; grassland converted into maize monoculture; arable land and arable land converted into grassland. *Burkholderia* isolates obtained on PCAT medium were grouped in 47 clusters using 16S ribosomal RNA gene based PCR-DGGE combined with BOX genomic fingerprinting (DGGE-BOX). The distribution of the isolates in the DGGE-BOX clusters was used to calculate the Shannon diversity index per treatment. Interestingly, we observed that the *Burkholderia* diversity was affected by changes in the agricultural management, since the highest diversity was observed in permanent grassland and in continuous arable land. In addition, the diversity tended to be higher in the rhizosphere than in the corresponding bulk soil. The use of species abundance models indicated that rhizosphere communities had more even distributions than communities collected from the bulk soil. Identification of isolates revealed that only 2% of these belonged to the *B. cepacia* complex and that the majority was assigned to either (1) new *Burkholderia* species or (2) *Burkholderia* species that had originally been isolated from soil. Isolates classified as *B. hospita*, *B. caledonica* and *Burkholderia* sp. 'LMG 22934' and 'LMG 22936' were found mainly in the arable land, while isolates belonging to *Burkholderia* sp. 'LMG 22929' and *B. phytofirmans* were associated with the grassland area. Another potentially new *Burkholderia* species, 'LMG 22932', was found in both areas, in close association with the maize rhizosphere.

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Keywords: *Burkholderia*; Agricultural management; Land use history; Diversity; Species abundance model.

1. Introduction

The genus *Burkholderia* has become popular in the agricultural sector as a result of the biocontrol abilities of some species (Cain et al., 2000; Hebbar et al., 1998). Additional beneficial traits, such as atmospheric nitrogen fixation (Estrada-De los Santos et al., 2001; Moulin et al., 2001; Estrada et al., 2002; Chen et al., 2003), plant growth promotion (Trân Van et al., 2000), and more recently, its potential in bioremediation (Master et al., 2002), increased the relevance of this genus for environmental purposes.

Similarly, in the clinical sector, the genus *Burkholderia* has gained importance in the last decade due to the pathogenicity of the so-called *B. cepacia* complex, which corresponds to several phenotypically similar species distributed over nine genomovars (Coenye et al., 2001c; Caballero-Mellado et al., 2004), for patients with cystic fibrosis (CF) (Mahenthalingam et al., 2002). The high virulence observed for certain species of the *B. cepacia* complex, mainly *B. cenocepacia* and *B. multivorans* (Speert et al., 2002), combined with the fact that *B. cenocepacia* has been found as a common plant-associated bacterium, isolated from rhizosphere and plant tissues (Balandreau et al., 2001), increased the concern that the environment might be a source of pathogenic strains (Lipuma, 2002). However, due to the lack of genetic markers of transmissibility or virulence, any distinction between environmental

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and clinical strains, or between pathogenic and non-pathogenic strains, remains, so far, impossible (Parke and Gurian-Sherman, 2001). Based on these facts, several biocontrol strains recently had their risk assessment modified by the US Environmental Protection Agency (Parke and Gurian-Sherman, 2001).

Burkholderia species have a broad natural distribution, occurring commonly in soil (Achouak et al., 1999), water (Vermis et al., 2003) and in association with plants (Bevivino et al., 1998; Balandreau et al., 2001; Moulin et al., 2001), fungi (Lim et al., 2003) and animals (Van Borm et al., 2002). However, little is known about the diversity of this group of organisms in these settings. In addition, for the reasons mentioned earlier, most of the attention has concentrated on the *B. cepacia* complex. Furthermore, since species belonging to the *B. cepacia* complex are found in close association with maize roots, representing up to 3.6% of the culturable bacterial population (DiCello et al., 1997), diversity studies have focused mainly on *B. cepacia* complex strains isolated from this plant (Bevivino et al., 1998; Dalmastri et al., 1999; Chiarini et al., 2000; Fiore et al., 2001). For instance, in a survey performed in maize fields with different management history, mainly *B. cepacia*, *B. cenocepacia*, *B. vietnamiensis* and *B. ambifaria* were isolated (Fiore et al., 2001). When different fields were compared (low versus high input), the highest degree of genomovar diversity was obtained from a field characterised by a low-input regime, to which only manure had been added (Fiore et al., 2001).

The impact of land use history and agricultural management on soil microbial community structure has been observed using both cultivation-based and direct approaches (Lupwayi et al., 1998; Steenwerth et al., 2002). However, not all microorganisms respond the same way, as it has for instance been shown that specific bacterial groups are differently affected by grassland management (Clegg et al., 2003). In a previous study, we observed that the diversity of the *Burkholderia* community, as determined by PCR-DGGE analysis of DNA from the rhizospheres of different plants, was affected mainly by land use history (Salles et al., 2004). Differences in the *Burkholderia* community structure due to agricultural management and plant species occurred to a lower extent as a result of the dominating influence of the history of agricultural use of the area (Salles et al., 2004). Given the effect of land use history on the diversity of the total *Burkholderia* community, the restricted information on the diversity of the culturable *Burkholderia* population and the increasing concern that human-pathogenic species might be prevalent in soil, our aim was to evaluate the impact of agricultural management on the diversity of culturable *Burkholderia* species and, in addition, to establish which species were correlated with the different management regimes. Therefore, rhizosphere and bulk soil samples, taken from field areas under different agricultural management, were plated on semi-selective PCAT medium and a large number of isolates was obtained. The isolates were

clustered according to their combined DGGE-BOX patterns and the data obtained were used to evaluate the diversity of the culturable *Burkholderia* populations and the distribution of species over the treatments.

2. Material and Methods

2.1. Isolation of *Burkholderia* strains from soil

To evaluate the diversity of *Burkholderia* species in soil under different agricultural management regimes, soil samples were taken from an experimental field located in the vicinity of Wageningen, the Netherlands. Briefly, this site was originally composed of a long-term (>50 y) permanent grassland (G) field that had been partially converted to agricultural land (A) around 24 years ago. In 2000, an experiment was established in both areas (G and A), in a randomized block design with three blocks per treatment and a range of treatments differing in agricultural management (van Elsas et al., 2002). For this study, two treatments were chosen: one consisting of monoculture of maize (*Zea mays* L.) and another one of permanent grassland (commercial mix, containing *Lolium perenne* as the main plant species). The maize plots received annual fertilisation, were tilled at 20 cm, and control of weeds was done mechanically, whereas the grassland plots were not fertilised or tilled. Grassland plots were sown at the beginning of the experiment (in the arable land) and the grass was cut once a year. Therefore, according to land use history and agricultural management, four treatments could be distinguished: (i) permanent grassland (which was kept as grassland) (G); (ii) an area under continuous agriculture, represented by arable land under maize monoculture (A–M); (iii) arable land converted to grassland (A–G); and (iv) permanent grassland which was turned into an agricultural field under maize monoculture (G–M).

In September 2003, after four growth cycles in the field (2000, 2001, 2002, 2003), soil samples were obtained from the four treatments. Bulk soil was obtained by combining 100 sub-samples, taken from the surface of each 10×10 m plot with a soil core sampler (3×10 cm, diameter×height). Rhizosphere soil was obtained by harvesting five maize plants, or grass, from five different locations per plot and gently shaking the roots in order to remove the loosely adhering soil. Twenty grams of roots with tightly-adhering soil (rhizosphere soil) or 10 g bulk soil were transferred, separately, to Erlenmeyer flasks containing 90 ml of sterile sodium pyrophosphate (0.1% NaPP in water) and washed gravel (10 g; approximately 30 small stones with length from 2–5 mm). After shaking the flasks for 30 min at 180 rev min⁻¹, a series of 10-fold dilutions in 0.85% NaCl was prepared and plated onto PCAT medium (composition: azelaic acid 2 g l⁻¹, tryptamine 0.2 g l⁻¹, MgSO₄·7H₂O 0.1 g l⁻¹, KH₂PO₄ 4 g l⁻¹, K₂HPO₄ 4 g l⁻¹, yeast extract 0.02 g l⁻¹, agar 15 g l⁻¹; pH 5.7) (Burbage et al., 1982)

supplemented with 100 mg l⁻¹ Delvolid (DSM, Delft, The Netherlands). Plates were incubated at 27 °C for 7 days, when approximately 30 colonies per replicate plot (15 colonies from bulk soil and 15 from rhizosphere soil) were transferred to new PCAT plates. After purification via re-growth of separate colonies on new PCAT plates, isolates were stored at -80 °C as cell suspensions in Trypticase Soya broth (Salles et al., 2002) containing 20% glycerol.

2.2. DNA extraction

DNA was obtained from the *Burkholderia* isolates by resuspending one colony per strain in a microcentrifuge tube containing 20 µl of sterile Milli-Q water. Cells were lysed by keeping the suspensions at 95 °C for 10 min, followed by cooling on ice for 10 min. Before performing the lysis, five mg of Chelex-100 resin (Sigma-Aldrich BV, Zwijndrecht, The Netherlands) was added to the microtubes to prevent inhibition of PCR amplification, given that compounds released by lysing cells are chelated by the resin and therefore removed from the suspension, by centrifugation at 1200 rev min⁻¹ for 5 min. The microcentrifuge tubes containing the crude lysates were stored at -20 °C.

2.3. PCR-DGGE analysis

Amplification of 16S rRNA genes of the *Burkholderia* strains was performed using the primers specific for the genus *Burkholderia* (Burk3-GC/BurkR) in a direct PCR, according to the methodology described by Salles et al. (2002). DGGE analysis was performed by using the PhorU2 system (Ingeny, Leiden, The Netherlands), in which gels with denaturing gradients of 50–60% of denaturant were run for 15 h at 100 V (Salles et al., 2002). After electrophoresis, the gels were stained with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, The Netherlands) and then photographed and digitised using the Imago compact apparatus (B&L System, Maarssen, The Netherlands).

2.4. BOX fingerprinting

Genomic fingerprintings of *Burkholderia* isolates was performed by PCR with primer BOX A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). Briefly, 1 µl of cell lysate was added to 24 µl of a mix containing 600 µmol l⁻¹ of each deoxyribonucleoside triphosphate, 3.75 mmol l⁻¹ MgCl₂, 10 mmol l⁻¹ Tris-HCl pH 8.3, 10 mmol l⁻¹ KCl, 500 nmol l⁻¹ of primer, and 2.5 U of *Taq* DNA polymerase Stoffel fragment (Perkin-Elmer). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc., Tilburg, The Netherlands). The thermal cycling program consisted of an initial denaturing step (95 °C for 7 min), followed by 30 cycles, each consisting of 30 s at 90 °C, 1 min at 95 °C, 1 min at 52 °C and 8 min at 65 °C. After the thermal cycling, there was a final extension step consisting

of 65 °C for 16 min, followed by cooling to 10 °C. The PCR products, expected to vary from 2 kb to 100 bp, were analysed by electrophoresis in 1.5% (wt/vol) agarose gel in 0.5×TBE buffer (Sambrook et al., 1989). Isolates that were not successfully amplified with the BOX A1R primer were subjected to a second new PCR reaction in which a higher or lower volume of crude cell lysate was added.

2.5. Banding pattern analysis

Digitised gel images obtained from DGGE analysis and BOX fingerprinting were converted, normalised and analysed separately with the Molecular Analyst software (version 1.61; Bio-Rad, Veenendaal, The Netherlands) (Rademaker et al., 1999). In order to achieve perfect normalisation of the gels, markers were loaded in at least 3 positions, in both DGGE and BOX gels. The marker used for DGGE gels consisted of partial 16S rRNA genes from four *Burkholderia* species as described by Salles et al. (2002). For normalising the BOX fingerprints, the molecular mass marker was used (Invitrogen BV, Breda, The Netherlands). In order to validate the normalisation procedure, identical profiles were run in different gels. After normalisation of each gel separately, the fingerprints were combined considering the DGGE profile as the 'master' gel. The similarity between the combined DGGE-BOX patterns was calculated using the Pearson or product-moment correlation coefficient, which is insensitive to the relative concentration of bands and to differences in the overall intensity of the profile (Rademaker et al., 1999). Cluster analysis was performed by applying the unweighted pair-group method using arithmetic averages (UPGMA) algorithm to the similarity matrix.

2.6. Identification of isolates

Species-level identification of *Burkholderia* isolates was achieved using a polyphasic approach. First, all isolates were examined using a *recA* based PCR test to identify putative *B. cepacia* complex bacteria (Mahenthiralingam et al., 2000). Isolates belonging to the *B. cepacia* complex were further identified using HaeIII-RFLP analysis of the resulting amplicons (Mahenthiralingam et al., 2000). All non-*B. cepacia* complex isolates were subsequently examined using comparative whole-cell protein electrophoresis as described before (Pot et al., 1994; Coenye et al., 2001c). Isolates were identified by comparing their whole-cell protein electrophoretic profiles with those present in a database comprising reference profiles of all known *Burkholderia* species (Vandamme et al., 1996; Coenye et al., 2001c); alternatively, several isolates were grouped into putative novel *Burkholderia* species because of the lack of similarity of their whole-cell protein profiles towards those of reference strains of all current *Burkholderia* species.

The exact phylogenetic position of representative isolates of putative novel species was determined by

cloning and sequencing their 16S rRNA genes. Amplification of the 16S rRNA genes from representative strains was performed with primers 27F (5'-AGAGTTTGATC [CA]TGGCTCAG-3') and 1378R (5'-CGGTGTGTA-CAAGGCCCCG GGAACG-3'), in 50 µl reaction mixtures containing 1 µl of cell lysate, 350 µmol l⁻¹ of each deoxyribonucleoside triphosphate, 300 nmol l⁻¹ of each primer, 1 × *TaqPlus* Precision buffer (Stratagene, Leusden, The Netherlands), and 2U of *TaqPlus* Precision polymerase mixture (Stratagene). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc. Tilburg, The Netherlands). The thermal cycling program consisted of an initial denaturing step (95°C for 2 min), followed by 35 cycles, each consisting of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C. After the thermal cycling, there was a final extension step consisting of 72 °C for 7 min, followed by cooling to 10 °C. The PCR products were analysed by electrophoresis in a 1% (wt/vol) agarose gel in 0.5 × TBE buffer (Sambrook et al., 1989). PCR products were then cloned in the pGEM-T easy vector, which was used to transform *Escherichia coli* strain JM109, according to the procedure recommended by the manufacturer (Promega Benelux, Leiden, The Netherlands). Plasmid extraction from randomly selected colonies was performed with the Wizard Plus SV miniprep DNA purification system (Promega Benelux). Sequencing was performed by using a ABI Prism automatic sequencer (Greenomics, Wageningen, The Netherlands).

2.7. Sequence alignment

Sequences generated in this study or recovered from the GenBank/EMBL database were aligned by using Clustal_X (Thompson et al., 1997), considering 1380 bp of the 16S rRNA gene. Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) based on distance estimation calculated by the method of Jukes and Cantor (1969). This analysis was performed with the TREECON program, version 1.3b (Yves van de Peer, Department of Biochemistry, University of Antwerp, Antwerp, Belgium).

2.8. Diversity measurements and statistical analysis

The cut-off value used to determine the level of similarity between DGGE-BOX clusters was tested by assessing its Discriminatory index (DI) (Coenye et al., 2002). DI determines the probability that two unrelated strains are placed into different groups and is calculated by using the Simpson index of diversity, which takes into account the number of clusters defined and their relative frequencies (Coenye et al., 2002).

The Shannon diversity index was determined as $H' = -\sum p_i \ln p_i$ (Magurran, 1988), where p_i is the proportion of individuals found in the i th DGGE-BOX cluster. To test for significant differences between

the Shannon diversity index obtained for each sample, a t test was performed according to the methodology described by Magurran (1988), as follows:

$$t = \frac{H'_1 - H'_2}{(\text{Var}H'_1 + \text{Var}H'_2)^{1/2}}$$

and the variance of H' was calculated using the equation:

$$\text{Var}H' = \frac{\sum p_i (\ln p_i)^2 - (\sum p_i \ln p_i)^2}{N} + \frac{S-1}{2N^2}$$

where N is the total number of individuals and S is the total number of DGGE-BOX clusters. The degrees of freedom were calculated according to the formula

$$df = \frac{(\text{Var}H'_1 + \text{Var}H'_2)^2}{(\text{Var}H'_1)^2/N_1 + (\text{Var}H'_2)^2/N_2}$$

where N_1 and N_2 are the total number of individuals in samples 1 and 2, respectively (Magurran, 1988).

Species abundance models were calculated according to the formulas described by Hill et al. (2003). The observed data were distributed among 5 abundance classes, divided according to the number of isolates per cluster, with upper boundaries of 2.5, 4.5, 8.5, 16.5 and 32.5 isolates per cluster. The observed distribution was then compared with the expected distribution obtained from the models by using a goodness-of-fit test (χ^2) (Magurran, 1988). Low χ^2 values (non-significant) indicate a good agreement between the observed and the expected data, i.e. that the model fits the distribution of the data (Magurran, 1988).

2.9. Nucleotide sequence accession numbers

The sequences generated in this study have been deposited in the GenBank database under accession numbers AY836218, AY836219 and AY949187 to AY949201.

3. Results

3.1. Enumeration and isolation of *Burkholderia* strains

The densities of the populations recovered from the semi-selective PCAT medium are shown in Fig. 1. Significant differences were observed between bulk and rhizosphere soils, the latter showing populations approximately at 100 times higher densities than the former, except for A–G. The highest densities were observed in the rhizospheres of maize grown in the arable land (A–M) and in those of grass collected from the permanent grassland (G). A total of 344 colonies from both bulk and rhizosphere soil (around 30 per treatment, 15 from bulk and 15 from rhizosphere) were recovered from PCAT plates. From these, 287 isolates (83.4%) were positive for the 16S rDNA PCR with *Burkholderia* genus-specific primers.

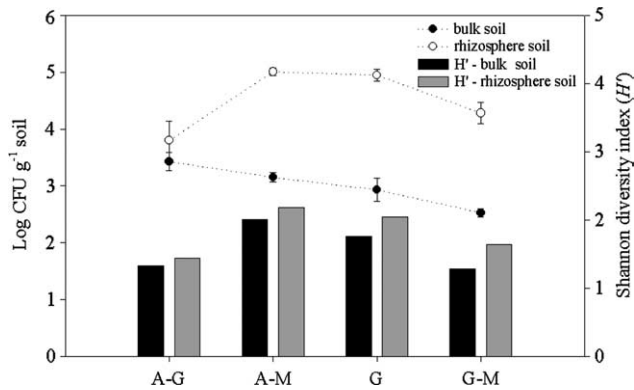


Fig. 1. Mean density of the *Burkholderia* population isolated on the semi-selective media PCAT, from bulk (●) and rhizosphere (○) soil collected from Arable land (A) and Grassland field (G), cultivated with maize (M) and grass (G). Mean value and standard error are shown. Bars indicate the Shannon diversity index (H') based on the number of DGGE-BOX types obtained from bulk (black bars) and rhizosphere (grey bars) *Burkholderia* soil populations.

3.2. DGGE-BOX banding pattern analysis

In order to characterise the culturable *Burkholderia* community in the different treatments, all 287 isolates were further characterised by BOX-PCR. The majority of the 287 *Burkholderia* isolates (254; 89%) were amplifiable with the BOX A1R primer, whereas the remainder (11%) was not further analysed. The BOX fingerprints of the 254 *Burkholderia* isolates were compared and grouped according to their similarity, yielding a set of 158 isolates that represented all BOX types. These selected isolates were further characterised by 16S rDNA based PCR-DGGE. Given that the PCR-DGGE method separates the isolates at approximately species level (Salles et al., 2002) while the BOX pattern has a resolution at strain level, the DGGE profile was considered the master pattern. The dendrogram generated by the combined DGGE-BOX pattern was divided in two main groups at 32% of similarity. One group contained most of the DGGE-BOX clusters, while the second group contained the *Burkholderia* isolates that had a lower G + C content in the 16S rRNA gene region amplified by the primers. Between 40–50% similarity, secondary and tertiary branches were formed for both groups and at 74%, the cut-off similarity value was set, generating 47 different DGGE-BOX clusters (Fig. 2) at a DI of 0.999.

3.3. Identification of the isolates

A total of 87 isolates representing the 47 DGGE-BOX clusters (1–5 isolates per cluster) was identified at the species level. Table 1 presents an overview of identification results from the total of 40 DGGE-BOX clusters assigned to one *Burkholderia* species, along with the method used for identification. The 40 clusters were distributed over 13 *Burkholderia* species, from which 8 represented putative novel species, referred to below as *Burkholderia* sp. 'LMG

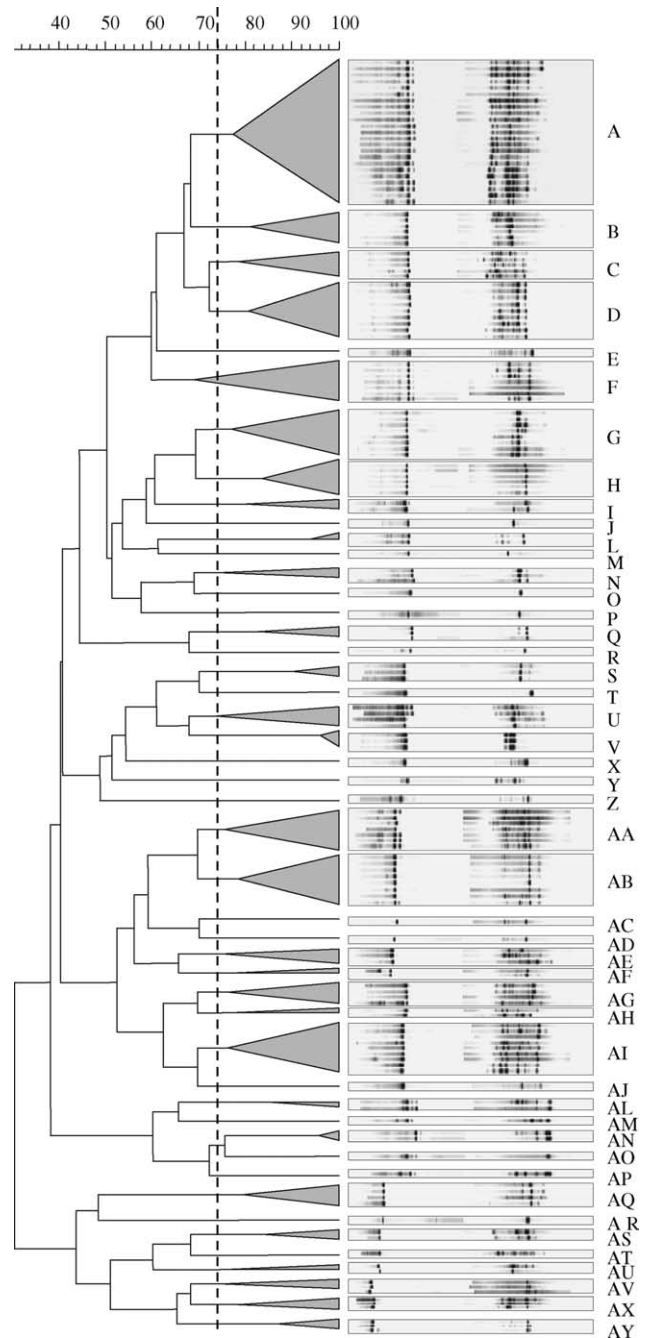


Fig. 2. Combined DGGE-BOX dendrogram, showing the distribution of *Burkholderia* isolates. Similarity was based on product-moment correlation coefficient (Rademaker et al., 1999). Cluster analysis was performed by applying UPGMA algorithm to the similarity matrix. For information about the distribution of isolates per cluster and their identification, see Table 1.

22929', *Burkholderia* sp. 'LMG 22932', *Burkholderia* sp. 'LMG 22934', *Burkholderia* sp. 'LMG 22936', *Burkholderia* sp. 'LMG 22946', *Burkholderia* sp. 'LMG 22940', *Burkholderia* sp. 'LMG 22945' and *Burkholderia* sp. 'LMG 22938'. Fig. 3 shows the whole-cell protein profiles of isolates representing the main groups detected in the present study, along with profiles of *Burkholderia* type or reference strains belonging to the same species, or, for the putative

Table 1

Distribution of the identified *Burkholderia* species per cluster. Only clusters assigned to one species are shown

Species	No of isolates	Cluster	No of isolates per treatment ^a				Method ^b	Reference ^c
			A–G	A–M	G	G–M		
<i>B. hospita</i>	71	A	6(0)	28(9)	–	1(0)	2 and 3	(Goris et al., 2002)
		AL	–	6(6)	–	–	2 and 3	
		AN	7(7)	–	–	–	2	
		AO	2(2)	–	–	–	2	
		AP	2(2)	–	–	–	2	
		F	18(18)	–	–	–	2 and 3	This study
		Y	–	1(0)	–	–	2	
<i>Burkholderia</i> sp. 'LMG 22929'	57	C	–	–	5(0)	–	2	
		D	–	–	16(0)	–	2 and 3	
		E	–	–	1(0)	–	2	
		G	–	1(0)	8(7)	2(0)	2 and 3	
		H	–	–	10(10)	–	2	
		I	–	–	–	2(1)	2	
		J	–	–	1(1)	–	2	
		L	–	1(0)	2(0)	–	2	
		M	–	–	1(1)	–	2	
		O	–	1(0)	–	–	2	
		P	–	–	2(2)	–	2	
		Q	–	–	4(0)	–	2	
<i>B. phytofirmans</i>	47	AA	2(0)	1(0)	–	9(2)	2 and 3	(Sessitsch et al., 2005)
		AB	–	1(1)	1(1)	13(13)	2 and 3	
		AI	–	3(1)	5(3)	4(1)	2	
		AT	–	–	1(0)	–	2	
		AJ	–	–	–	7(7)	3	This study
<i>Burkholderia</i> sp. 'LMG 22932'	22	B	–	4(0)	–	18(0)	2 and 3	
<i>Burkholderia</i> sp. 'LMG 22934'	13	AV	1(1)	1(1)	–	1(1)	2	This study
		AX	3(0)	1(1)	–	–	2 and 3	
		AY	1(1)	4(4)	–	–	2 and 3	(Coenye et al., 2001b)
		AR	–	–	1(1)	–	2	
<i>B. caledonica</i>	6	AC	1(1)	–	–	–	2	
		AD	–	1(1)	–	–	2	
		S	–	3(0)	–	–	2 and 3	This study
		T	–	1(0)	–	–	2	
<i>Burkholderia</i> sp. 'LMG 22936'	4	AQ	–	4(3)	–	–	2 and 3	This study
<i>Burkholderia</i> sp. 'LMG 22946'	3	V	–	–	3(0)	–	2 and 3	
<i>Burkholderia</i> sp. 'LMG 22940'	2	AS	1(0)	1(0)	–	–	2 and 3	This study
<i>Burkholderia</i> sp. 'LMG 22945'	1	R	–	–	1(0)	–	2 and 3	
<i>Burkholderia</i> sp. 'LMG 21262'	1	X	–	1(1)	–	–	2 and 3	(Goris et al., 2002)
<i>Burkholderia</i> sp. 'LMG 22938'	1	Z	–	1(0)	–	–	2	
<i>B. pyrrocinia</i>	1	AM	–	–	–	1(0)	1 and 3	(Viallard et al., 1998)

^a Number in brackets indicates isolates obtained from bulk soil.^b Methods used for identification: 1, RFLP of the *recA* gene; 2, comparison of the whole cell protein profiles; 3, sequencing of the partial 16S rRNA gene.^c Indicates the reference where the species is described.

novel species, belonging to their nearest phylogenetic neighbours. A phylogenetic tree based on the 16S rRNA gene is shown in Fig. 4, indicating the position of some representative isolates described in Table 1 in the genus *Burkholderia*.

The species most frequently isolated was *B. hospita* (Goris et al., 2002), representing 28% of the isolates, which were distributed over seven DGGE-BOX clusters. Interestingly, 98.6% of the *B. hospita* isolates were recovered from the arable land carrying maize or grass (A–M and A–G)

(Table 1). Twenty-two percent of the *Burkholderia* isolates were identified as *Burkholderia* sp. 'LMG 22929' and about 95% of these isolates were obtained from the plots with grassland history (G and G–M). In fact, this group was clearly correlated with permanent grassland (G), which contained 88% of the isolates. In addition, this was the most diverse *Burkholderia* species, being represented by 12 DGGE-BOX clusters. Another abundant group of isolates (19%), spread over five clusters, was identified as *B. phytofirmans*, a new *Burkholderia* species originally

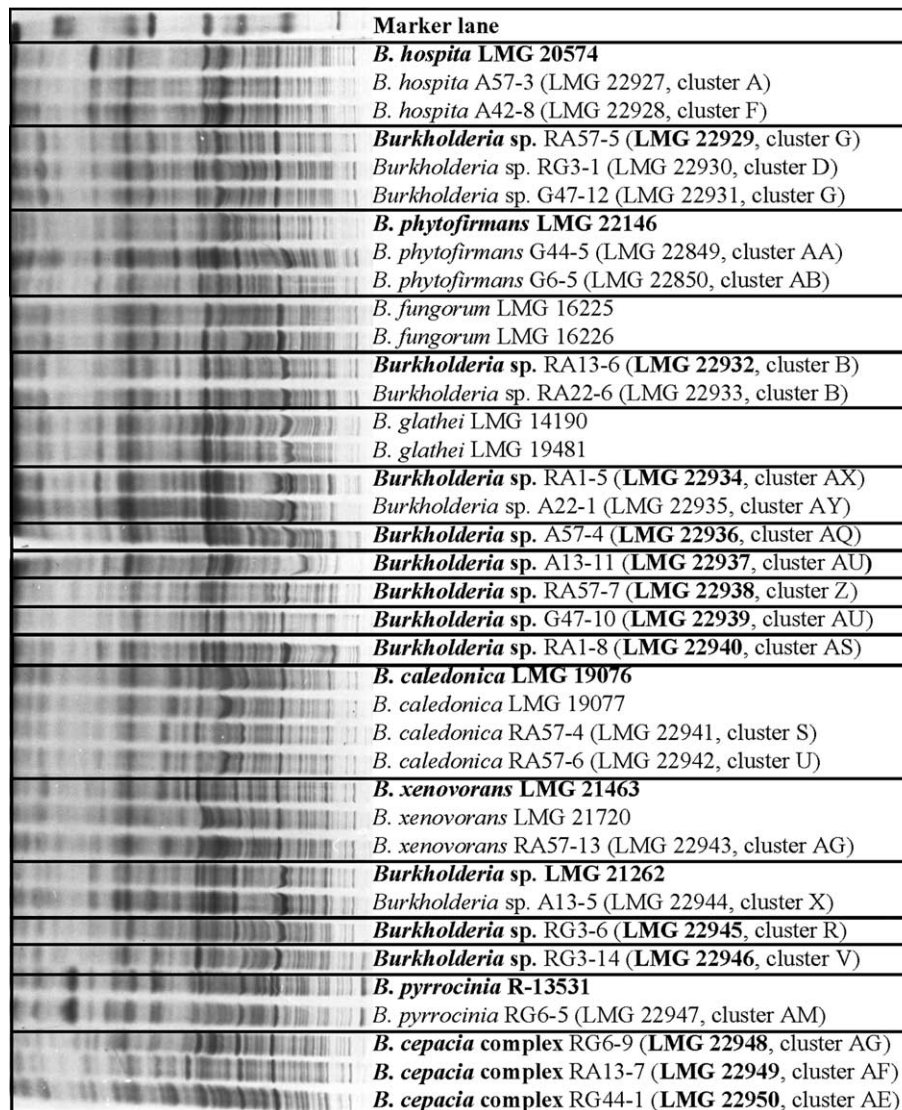


Fig. 3. Whole-cell protein electrophoretic patterns of the *Burkholderia* isolates from the present study and of reference strains. The molecular weight markers used (top lane) were (from left to right) lysozyme (molecular weight, 14,500), trypsin inhibitor (20,000), trypsinogen (24,000), carbonic anhydrase (29,000) glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumin (45,000), bovine albumin (66,000) and beta-galactosidase (116,000).

isolated from onion infected with arbuscular mycorrhiza (Sessitsch et al., 2005). This species was also mainly isolated from the plots with grassland history (85%) and the highest number of isolates was recovered from grassland converted into maize monoculture (G–M, 70%). *Burkholderia* sp. ‘LMG 22932’, corresponding to a single cluster containing 9% of the isolates, was highly prevalent in the maize rhizosphere and contained isolates from plots currently under maize, but with different land use history (A–M and G–M). *Burkholderia* sp. ‘LMG 22934’ represented 5% of the isolates, spread over four clusters, and was mainly recovered from the arable land, currently under different crops (A–G and A–M). Two percent of the isolates, which were distributed over four clusters, were assigned to *B. caledonica* (Coenye et al., 2001b). These isolates were mainly obtained from the rhizosphere of maize plants collected from the arable field (A–M). *Burkholderia* sp.

‘LMG 22936’ (2% of the isolates) was mainly isolated from the bulk soil of the arable area under maize monoculture (A–M) and was represented by clusters AQ. Cluster V contained 1% of the isolates, which were obtained from permanent grassland (G) and identified as *Burkholderia* sp. ‘LMG 22946’. Cluster AS was identified as *Burkholderia* sp. ‘LMG 22940’ and represented by 1% of the isolates, mostly obtained from rhizosphere soil of the arable land. Cluster R encompassed one isolate, called *Burkholderia* sp. ‘LMG 22945’, which was isolated from the permanent grassland (G). Both clusters X and Z were represented by one isolate each, obtained from the bulk and rhizosphere soil of arable land under maize monoculture (A–M), respectively. The isolate from cluster X belonged to a novel *Burkholderia* species described by Goris et al. (2002). The latter novel species is referred to below as *Burkholderia* sp. ‘LMG 21262’. The isolate from cluster Z was assigned to

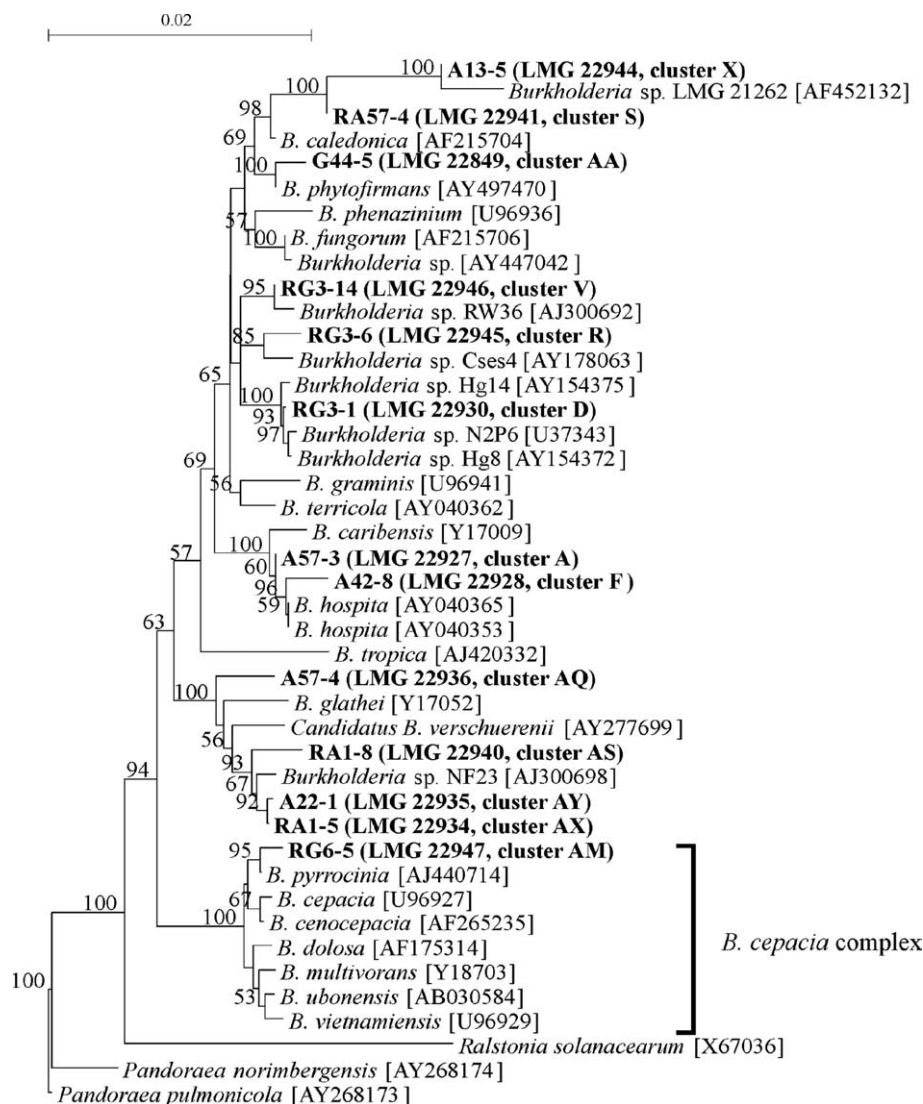


Fig. 4. Phylogenetic tree showing the relationship between some *Burkholderia* species and the isolates described on Table 1. The tree was constructed based on 1384 bp of the 16S rRNA gene, using the neighbour-joining method (Saitou and Nei, 1987). A bootstrap analysis was performed with 100 repetitions, and only values above 50 are shown. The accession number of each strain is enclosed in square brackets.

Burkholderia sp. 'LMG 22938'. Cluster AM, which also comprised one isolate recovered from the rhizosphere of maize growing in the grassland plot (G–M), was assigned to *B. pyrrocinia* (Vandamme et al., 2002b).

Although the majority of the DGGE-BOX clusters were assigned at species level (Table 1), seven clusters comprised isolates belonging to more than one *Burkholderia* species (data not included in Table 1). Cluster N comprised four isolates obtained from the rhizosphere of the permanent grass (G). Three of these were assigned to *B. phytofirmans* and one to *Burkholderia* sp. 'LMG 22929'. Similarly, cluster AH was represented by two rhizosphere isolates recovered from the area under permanent grassland (G), which were identified as *B. phytofirmans* and *Burkholderia* sp. 'LMG 22929'. The isolates grouped in cluster U were identified as *B. caledonica* (two isolates) and *B. hospita* (four isolates) and were recovered from the rhizosphere of

maize or grass, grown in the arable land (A–M and A–G). Cluster AG comprised four isolates: two were identified as *B. caledonica* and one as *B. xenovorans* (Goris et al., 2004). The fourth isolate (*Burkholderia* sp. 'LMG 22948') was identified as belonging to the *B. cepacia* complex by protein profile, while sequencing analysis of the 16S rRNA gene indicated 97% similarity with *Burkholderia* sp. strain RP007 (accession number AF061872; Laurie, A.D. and Lloyd-Jones, G., unpublished). These were all obtained from the rhizosphere of maize, growing in the arable land (A–M; *B. caledonica* and *B. xenovorans*) or in the grassland (G). Four isolates collected from the maize rhizosphere (G–M) represented cluster AE. Of these, one was identified as *B. phytofirmans* and the remaining three as belonging to *B. cepacia* complex. The latter strains, 'LMG 22950', were characterised by a novel *HaeIII-recA* restriction profile and therefore, their precise taxonomic status within

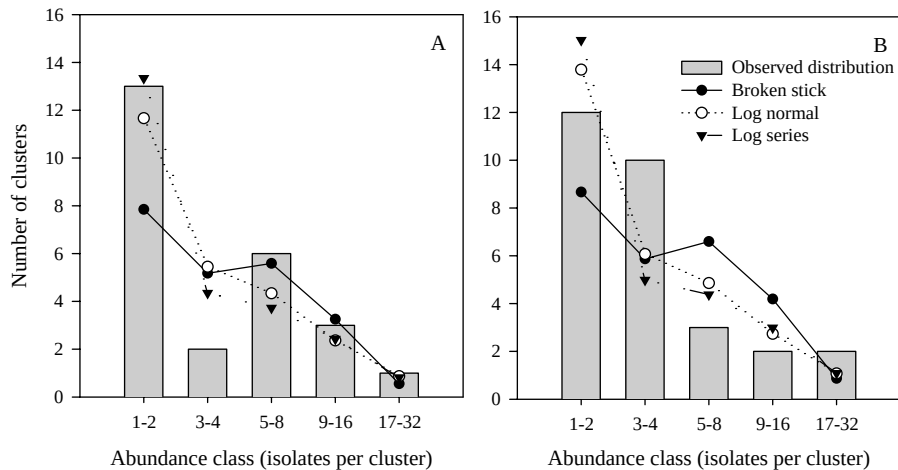


Fig. 5. Observed and predicted distributions of the DGGE-BOX clusters in bulk (A) and rhizosphere soil (B). Species abundance classes were calculated according to the formulas described by Hill et al. (2003).

the *B. cepacia* complex requires further taxonomic analyses. Cluster AF comprised three isolates from the rhizosphere of maize growing in the arable land (A–M). Two of these were identified as *B. hospita*, while the third one was identified as belonging to the *B. cepacia* complex by protein profile. Further taxonomic work is required to address the status of this isolate ('LMG 22949'), since amplification of the *recA* gene was not successful. According to the partial sequencing of the 16S rRNA gene, this isolate was most similar to *B. vietnamiensis* strain LMG 10929 (accession number AF097534) (Trâ Van et al., 1996). Cluster AU comprised two isolates, identified as *Burkholderia* sp. 'LMG 22939' and 'LMG 22937', which were isolated from bulk soil of permanent grassland and arable land under maize monoculture, respectively.

3.4. Diversity measurements

The distribution of the *Burkholderia* strains in the 47 clusters defined by DGGE-BOX fingerprinting was used to evaluate the diversity of the community as related to the treatments. Fig. 1 shows that the Shannon diversity index (H') for bulk and rhizosphere soils displayed the same trend, with the highest values being obtained for arable land under maize monoculture (A–M) and the lowest for arable land converted to grassland (A–G). In addition, the Shannon diversity index obtained for the *Burkholderia* community associated with rhizosphere soil tended to be higher than for that of the bulk soil, however, this difference was significant ($P=0.01$) only for the soil collected from the areas with grassland history (G and G–M) (Fig. 1). The *Burkholderia* communities associated with the rhizosphere of grass collected from the permanent grassland (G) and of maize growing in the arable land (A–M) were the most diverse according to the Shannon diversity index ($P=0.001$) (Fig. 1). When comparing the *Burkholderia* communities of the bulk soils, the lowest diversity indices were found in

the arable land converted to grassland (A–G) and grassland converted to maize monoculture (G–M) ($P=0.001$) (Fig. 1).

To compare the effects of the plant rhizospheres on the diversity of *Burkholderia* communities, all isolates obtained from rhizosphere or bulk soil samples were pooled, generating two data sets (bulk and rhizosphere soil) that were divided in five abundance classes (Hill et al., 2003). Three species abundance models, ranging downward in equitability from 'broken stick' to 'log series' (Hill et al., 2003) were tested and compared with the observed distributions. Although some differences in the distribution could be observed between the rhizosphere and the bulk soil community (mainly in the intermediate classes) (Fig. 5), all models fitted the observed distribution to some extent, for both data sets. The distribution of the bulk soil community could be described by the broken stick model, the truncated log normal and the log series model with probabilities of $P=0.30$, 0.50 and 0.70 , respectively. The rhizosphere community data could be described by these models with probabilities of $P=0.10$, 0.30 and 0.20 , respectively.

4. Discussion

4.1. *Burkholderia* isolation from soil samples

To evaluate the culturable *Burkholderia* communities in soils under different agricultural management, organisms able to grow on the semi-selective PCAT medium were assessed. PCAT has been widely used for assessing the diversity of species belonging to the *B. cepacia* complex in the environment (DiCello et al., 1997; Pallud et al., 2001; Richardson et al., 2002; Dalmastrri et al., 2003), although most *Burkholderia* species are able to grow on it (Viallard et al., 1998). By using genus-specific primers, we indeed observed that the majority of PCAT colonies belonged to the genus *Burkholderia*, whereas only 2% of the isolates

belonged to the *B. cepacia* complex. The fraction of isolates recovered from PCAT that belongs to the *B. cepacia* complex is known to be variable and dependent on the environmental source, ranging from around 74% (DiCello et al., 1997; Tabacchioni et al., 2000) when roots are blended prior to dilution and plating, to below detection when bulk soil from a maize field was taken as sample (Pallud et al., 2001). The differences may be attributed to the fact that, when macerated roots are used for dilution, not only the rhizosphere community is being analysed, but also the rhizoplane and the endophytic communities. These results indicate that PCAT is suitable for the enumeration and isolation of *Burkholderia* species, but not specifically for species from the *B. cepacia* complex. However, since the list of species belonging to the genus *Burkholderia* has been altered several times since the work performed by Viallard et al. (1998), with new species being added annually, more studies are necessary to verify which *Burkholderia* species are able to grow on PCAT medium.

4.2. *Burkholderia* species associated with agricultural management regimes

In the present study, we used a polyphasic strategy to identify *Burkholderia* isolates to the species level, in order to assess whether the clustering based on the DGGE-BOX approach was suitable to determine the *Burkholderia* diversity. Several studies of the genus *Burkholderia* confirmed the correlation between the whole-cell protein profile similarity and DNA–DNA hybridisation, the latter being the standard for species delineation in bacterial taxonomy (Coenye et al., 1999, 2001a, 2001b; Goris et al., 2002; Goris et al., 2004; Vandamme et al., 1996, 1997, 2002a). Only for some of the *B. cepacia* complex species which are very closely related, whole-cell protein profiles are too similar to allow unequivocal species differentiation (Coenye et al., 2001c) and, therefore, alternative molecular approaches have been elaborated (Mahenthiralingam et al., 2000). The identification results showed that isolates of most of the DGGE-BOX clusters could be identified at species level. Forty-one percent of the isolates were distributed over eight potentially new *Burkholderia* species, which, in general, were closely related to species commonly isolated from the environment. However, a full taxonomic characterisation of these species is beyond the scope of the present study.

By evaluating the distribution of the *Burkholderia* species among the treatments, we observed that they could be divided in two groups, according to the land use history of the area. *Burkholderia* isolates identified as *B. hospita* or *Burkholderia* sp. ‘LMG 22934’ seemed to be well adapted to changes in agricultural management, since they occurred in both arable land under maize monoculture and arable land converted to grassland. Since *B. hospita* has originally been isolated from agricultural soil as a transconjugant that had received plasmids pJP4 or pEMT1 (Goris et al., 2002), its

plasticity may be due to its ability to acquire plasmids from other soil bacteria. *B. caledonica* and *Burkholderia* sp. ‘LMG 22936’ occurred mainly in the arable land under maize monoculture, although the former was mainly found in association with maize roots, while the latter was also found in the bulk soil. This result is in agreement with the original report of *B. caledonica*, which was first isolated from rhizosphere soil in Scotland (Coenye et al., 2001b). The isolates obtained from grassland were mostly distributed among two species, *Burkholderia* sp. ‘LMG 22929’ and *B. phytofirmans*. The former isolates were highly linked to the permanent grassland whereas the latter was found mainly in the grassland converted to maize monoculture (G–M). *B. phytofirmans* has been recently described as a powerful plant-growth promoting bacterium (Sessitsch et al., 2005). However, additional tests should be performed in order to verify if plant-growth promotion is a common trait within this species. The only organisms that might be correlated with agricultural management regime were affiliated with *Burkholderia* sp. ‘LMG 22932’. This taxon was highly associated with the maize rhizosphere, occurring in maize planted both in former grassland (G–M) and in arable land (A–M).

4.3. Implications of agricultural management regimes for the diversity of culturable *Burkholderia* species

Rhizosphere bacterial communities seem to have a higher level of culturability than bulk soil communities due to the enhanced substrate availability observed around the roots (de Ridder-Duine et al., 2004). Indeed, the *Burkholderia* population densities present in the rhizosphere were 100-fold higher than those in the bulk soil in all treatments, except A–G. Due to the differences in cell densities, the *Burkholderia* community associated with bulk and rhizosphere soils were compared regarding the distribution of their clusters within each of these two subsets of the *Burkholderia* community, by fitting the observed abundance to species abundance models. Most likely as a result of the size of our data set and the low number of abundance classes, we were not able to clearly specify the model that best fitted the data set. Nevertheless, abundance models still address the distribution of the population and can be recommended even when coverage is low (Hill et al., 2003). According to the species abundance models, the *Burkholderia* community associated with rhizosphere soil (log-normal distribution) was more even than the community in the bulk soil (log series). The log series abundance model represents the least equitable distribution tested. It tends to occur when only one or few factors dominate the ecology of the community (Magurran, 1988) and few groups are abundant. In the case of the bulk soil, the factors determining community composition could be the agricultural practices applied to the fields. In the rhizosphere soil, the model that best fitted the distribution of the *Burkholderia* community was the truncated log-normal

model, which may originate from a community that has been affected by random variation of many independent factors (Hill et al., 2003). In our case, these random factors could be correlated with differences in quality and quantity of root exudation, in addition to agricultural management.

It is generally accepted that the rhizosphere community is a subset of the bulk soil community and is, therefore, less complex (Duineveld et al., 2001). In this study, however, the diversity of the culturable *Burkholderia* communities associated with the treatments (estimated by comparing the Shannon diversity index) was consistently higher in the rhizosphere than in the bulk soil, irrespective of land use history or agricultural management. Since only the culturable fraction of the *Burkholderia* community was assessed, one can speculate that the higher diversity observed in the rhizosphere was due to a positive effect of plant roots on the ability of the isolates to grow on the selective medium used. Furthermore, comparison of the Shannon diversity indices of the culturable *Burkholderia* communities associated with the treatments revealed that the *Burkholderia* diversity at the culturable level was affected mainly by changes in the agricultural management. The treatments with lower diversity indices were those subjected to a new agricultural management (A–G and G–M), suggesting that the conversion of arable land to grassland and vice versa led to a decrease in diversity. This result indicates that the impact of agricultural practices (fertilisation, tillage, etc.) applied to certain agricultural management regimes, can be greater than the crop used in that management. Moreover, the changes in soil management may have induced the selective outgrowth of specific *Burkholderia* types or species, decreasing the evenness and species abundance. Different plant species can select different portions of the microbial community (Smalla et al., 2001; Garbeva et al., 2004), but care should be taken when comparing plants growing in soils with different land use history or soils that went through recent changes in management. If we had considered only the arable land, we would have concluded that the *Burkholderia* community associated with maize (maize monoculture) was more diverse than the one associated with grass (grassland). On the other hand, by sampling only the plots derived from the permanent grassland, the opposite conclusion would have been achieved.

4.4. Conclusions

The diversity of the culturable *Burkholderia* community was mainly affected by the changes in the agricultural management, regardless of the direction of this change (towards more exploitative or more conservative management). Moreover, the culturable *Burkholderia* community associated with the rhizosphere was more even than the one associated with the bulk soil. Identification of the isolates showed that only 2% belonged to the *B. cepacia* complex and that the majority of the species was assigned to either

new *Burkholderia* species or to *Burkholderia* species commonly found in the soil.

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